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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)  
The overall goal of our research is to investigate the correlation between BRCA1-linked breast cancers and the Estrogen Receptor (ER)-negative phenotype. Two possible mechanisms have been investigated. The first was to evaluate the degree of DNA methylation in the region of the ER promoter in BRCA1-linked breast cancers. Methylation of CpGs within the ER promoter has been implicated as an operative mechanism of repressed expression in some cell lines and tumor specimens. The second approach was to utilize ER promoter constructs in transient transfection experiments to determine whether expression of BRCA1 affected transcriptional activity at this promoter. We analyzed a panel of human breast cancer cell lines and a collection of specimens from ER-negative breast cancers for CpG methylation of the ER promoter. The conclusion from those experiments is that ER-negative BRCA1-linked breast cancers are more highly methylated within the ER promoter region than are ER-negative non BRCA1-linked tumors. In transfection experiments of ER promoter constructs into two immortalized mammary epithelial cell lines, we determined that BRCA1 can transactivate the ER promoter. The portion of the ER promoter mediating this effect of BRCA1 was localized to between 62 bp and 171 bp downstream of the main transcription start site.

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## **Introduction:**

The overall goal of the research supported by this award was to investigate the correlation between *BRCA1*-mutated breast cancers and the Estrogen Receptor (ER)-negative phenotype. Although most sporadic breast cancers are ER-positive, studies have consistently shown that the vast majority of *BRCA1*-linked breast cancers are ER-negative (1-3). In sporadic cancers lacking ER expression, decreased expression of ER mRNA has been noted, without genomic DNA mutations in the ER gene (4-8). Two possible mechanisms by which breast cancers arising in the absence of functional *BRCA1* are more likely than not to be ER-negative have been investigated. The first (Task I) was to evaluate the degree of DNA methylation in the region of the ER promoter in *BRCA1*-linked breast cancers. Methylation of CpGs within the ER promoter has been implicated as an operative mechanism of repressed expression in some cell lines and tumor specimens. The second approach (Task II) was to utilize ER promoter constructs in transient transfection experiments to determine whether expression of *BRCA1* affected transcriptional activity at this promoter.

## **Body of Report:**

### **Task I**

In the annual report from Yr 1 (dated January 2001) we detailed our studies developing and standardizing assays of CpG methylation at the ER promoter that we planned to apply to a collection of ER-negative breast cancers. For this, we used a panel of ER-positive and ER-negative human breast cancer lines, one of which was the ER-negative line derived from a *BRCA1* mutation carrier (HCC1937). We initially conducted the analysis by two assays. First, we used the so-called Methylation Specific PCR (MSP) assay of bisulfite-treated DNA, based on the use of PCR primers which selectively target selected clusters of CpGs, taking advantage of the fact that the pre-PCR bisulfite treatment will change unmethylated CpGs into TpGs, while leaving methylated CpGs unaltered. Data from the ER-positive line MCF7 confirmed prior reports of an unmethylated ER promoter (see Table 1 on pg 5 of the Yr1 report. Our findings with the ER-negative cell lines suggested that *BRCA1*-linked breast cancers (represented by the HCC1937 line) are less methylated at the ER promoter than *BRCA1*-wildtype ER-negative breast cancer cells (see Figure 1 in our the appended publication, which is *in press* in the journal *Oncogene*). As a complementary assay, one not dependent upon bisulfite treatment and modification of the DNA, we applied the assay of Iwase *et al.* in which genomic DNA was first digested with the methylation-sensitive enzyme HpaII, then amplified with primers that span the restriction cut site (9). Experiments with the cell line DNA yielded results consistent with the MSP analyses.

We then compared 18 ER-negative breast cancers from women with documented germline mutations in *BRCA1* to a collection of 18 ER-negative breast cancers not linked to *BRCA1*. Specimens came from our own institution, as well as from collaborators at Memorial Sloan Kettering, Cleveland Clinic, and University Hospital in Lund, Sweden. DNA was extracted from the specimens (see Methods section in the appended publication) and analyzed by both the HpaII digest and MSP-based assays. As shown in the column labeled 'HpaII Digest' in Table I in the Yr2 report (pg 5, submitted February 2002), significant methylation was evident in the *BRCA1*-linked specimens (94%). Among the non *BRCA1*-linked group 81% showed methylation, consistent with published data (9). While the difference

between the *BRCA1*-linked and non *BRCA1*-linked groups was not statistically significant ( $p > 0.2$ ), these data suggested that our findings with the HCC1937 cell line (i.e. no methylation of the ER promoter) are not representative of primary *BRCA1*-linked breast cancers.

MSP analysis of DNA from the patient specimens, using the ER1 primer pair as described in (10), was also presented in Table I in the Yr2 report. As a semi-quantitative measure of the relative abundance of methylated and unmethylated DNA at the ER1 primer binding sites, we amplified bisulfite-treated DNA with the methylated DNA-specific primer pair as well as with the degenerate primer pair in parallel amplification reactions and compared the relative intensity of the resulting PCR products on an agarose gel. In separate reactions utilizing synthesized templates representing methylated and unmethylated ER1 sequence, we determined that PCR products of equal intensity with these primer pairs resulted when the methylated DNA template constituted ~10% of the total, probably reflecting a lower efficiency of amplification with the degenerate primers (not shown). We observed that whereas only 1 out of 12 samples in the non *BRCA1*-linked group produced a PCR band of greater intensity with the methylated DNA-specific primers than with the degenerate primers, half of the *BRCA1*-linked group (4 out of 8) yielded a PCR band of greater intensity with the methylated DNA-specific primers than with the degenerate primers. This represents a significant difference between the two groups ( $p = 0.035$  by Chi-Square analysis), consistent with a higher level of CpG methylation being present in the cancers from *BRCA1* mutation carriers.

These data indicated that the hypothesis that *BRCA1*-linked breast cancers will be notably unmethylated, as was the case with the HCC1937 cell line derived from a *BRCA1* mutation carrier, is probably incorrect. Indeed, the MSP data with the ER1 primer pair suggested the opposite hypothesis. A significant limitation of these data, however, is that they reflect methylation status at only a few CpG sites (those within the HpaII restriction sites flanked by the specific PCR primers, and those within the MSP primer sequences).

At this point in our work, we decided to devote the remainder of the DNA extracted from patient specimens to examination of methylation status by sequence analysis of bisulfite-treated DNA. To develop and standardize the methodology, we first analyzed DNA from our panel of four ER-negative human breast cancer cell lines. The ER1 and ER5 regions were amplified from bisulfite-treated DNA with degenerate primers, such that both methylated and unmethylated template DNA would be co-amplified. The resulting PCR products were then directly sequenced, thereby providing data on 25 CpGs located within the amplified regions. Conversion of the non CpG cytosines was >95%, indicating that incomplete bisulfite treatment was not the reason for heterogeneity of methylation noted in the MSP analysis. Figure 2 in the appended publication shows the percent methylation at each CpG site in our panel of ER-negative cell lines. In support of the MSP data reported in last year's report, MCF10A cells showed the highest level of methylation while HCC1937 cells showed the lowest level of methylation across the ER1 and ER5 regions. MDA-MB-231 cells, showing a heterogeneous MSP signal, were confirmed by sequencing to have an overall percent methylation between that of MCF10A cells and HCC1937 cells. 184B5 cells were shown by sequencing, as with MSP, to be highly methylated in ER1, but largely unmethylated in ER5. As a measure of methylation across the ER1 and ER5 sequenced regions, we averaged the percent

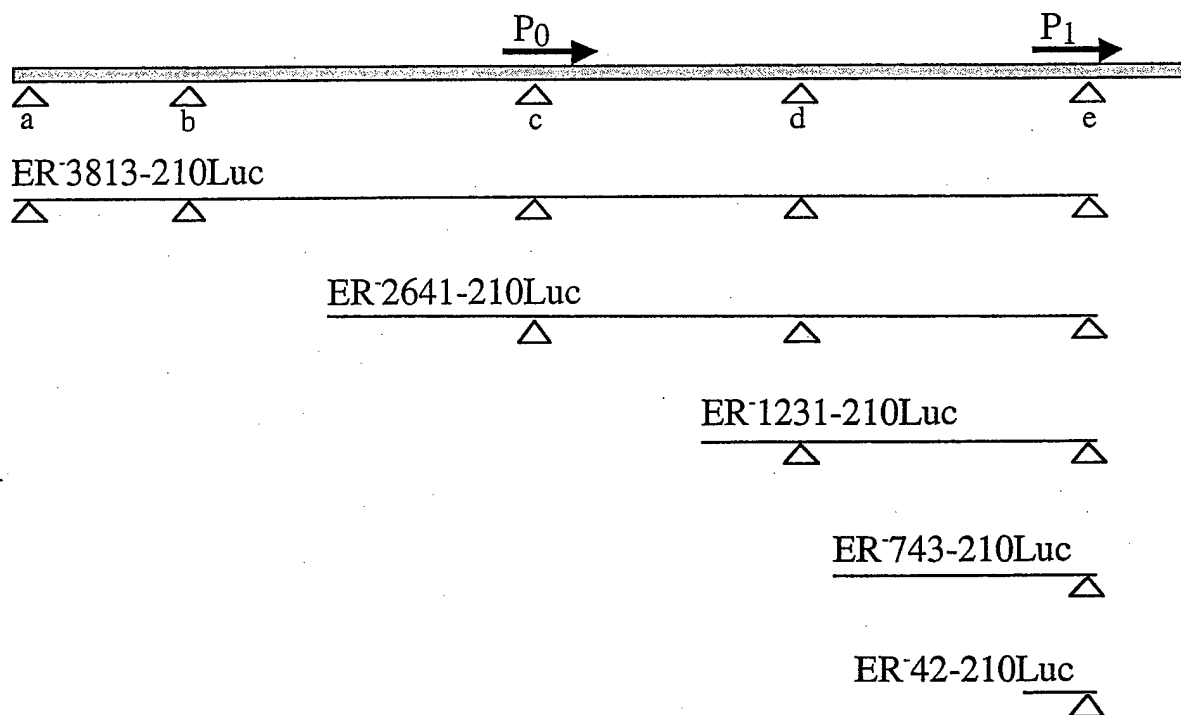
methylation of all CpG sites. The average percent methylation was 71% in MCF 10A cells, 11% in HCC1937 cells, 39% in MDA-MB-231 cells and 32% in 184B5 cells. Of note, only the 184B5 cells demonstrated markedly different degrees of methylation between the ER1 and ER5 regions (56% in ER1 vs. 1% in ER5).

Having established the sequence-based assay of CpG methylation status in these regions of the ER promoter, bisulfite-treated DNA from the tumor specimens was amplified with degenerate primers for both the ER1 and ER5 regions, and then analyzed by DNA sequencing to ascertain the percent methylation at each of 25 CpG sites (see Materials and methods in the appended publication for a full description of this assay). Figure 3 in the appended publication illustrates the average methylation at each CpG for the non *BRCA1*-linked and *BRCA1*-linked breast cancer groups. Methylation was higher at most CpG sites in the *BRCA1*-linked breast cancers as compared to the non *BRCA1*-linked breast cancers in both the ER1 and ER5 regions (Panels A and B of Figure 3, respectively). The overall average percent methylation was 25% among non *BRCA1*-linked cancers and 40% among *BRCA1*-linked cancers ( $p = 0.0031$ ). Specifically within the ER1 regions, the overall average percent methylation was 24% and 43% ( $p = 0.0041$ ) respectively, and within ER5, 27% and 39% ( $p = 0.0094$ ) respectively. There were no significant differences in the overall average percent methylation between the ER1 and ER5 regions in the primary specimens. Of note, 5 of the 25 examined CpGs, located at positions 62, 68, 121, 129 (Panel A), and 423 (Panel B), exhibited a greater than 2-fold increase in average methylation in the *BRCA1*-linked group compared to the non *BRCA1*-linked group ( $p < 0.03$ ).

Of the 25 CpG sites investigated by sequencing, 8 demonstrated significantly more methylation in the *BRCA1*-linked specimens compared with the non *BRCA1*-linked specimens ( $p < 0.03$  for each CpG), 5 of which demonstrated no overlap in the 95% confidence intervals ( $p < 0.01$  for each CpG). Data from the individual specimens for the panel of eight *discriminative* CpG sites are presented in Figure 4 of the appended publication. It is evident from examination of the individual data points that clear outliers do exist for both the non *BRCA1*-linked and *BRCA1*-linked groups.

## Task II

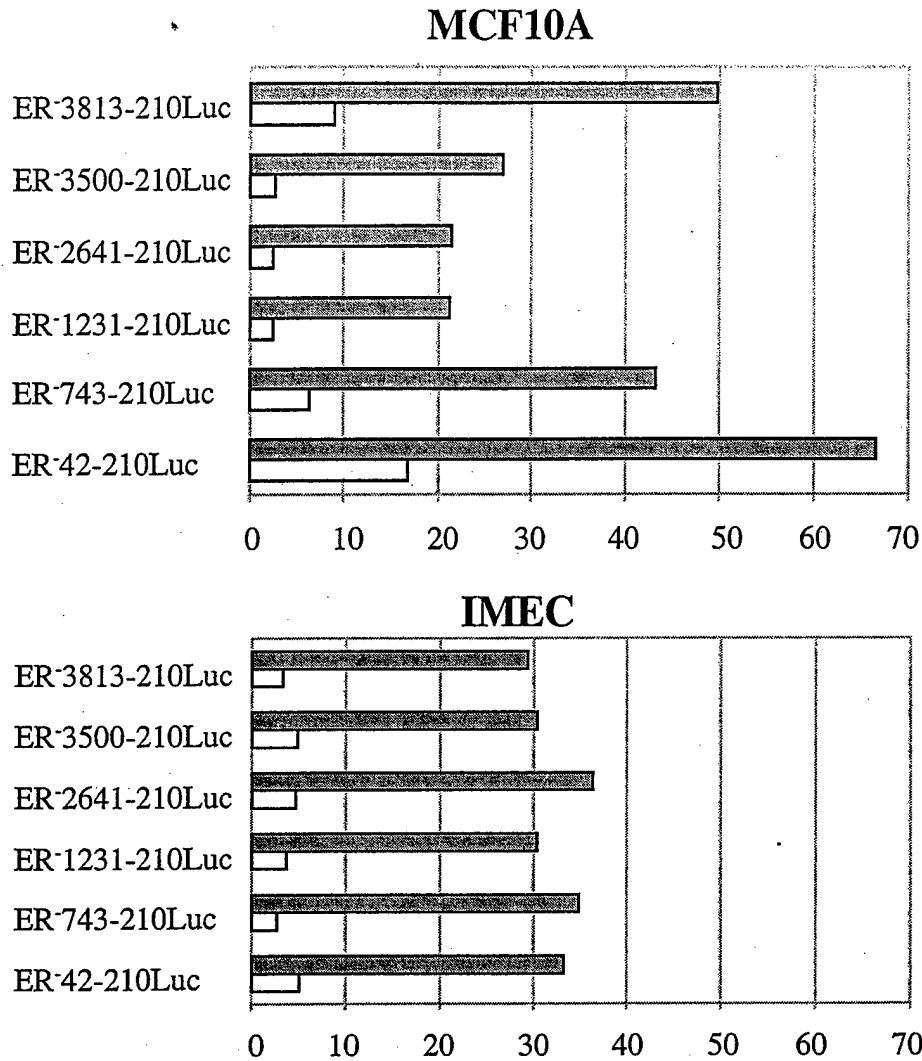
The most recent period of funding has been largely devoted to a test of the hypothesis that *BRCA1* exhibits transcriptional activation activity towards the ER promoter. To prepare for these experiments, we have prepared an ER promoter construct driving expression of luciferase. First, we obtained as a generous gift a construct from Dr. R.J. Weigel in which ~3.5kb of sequence upstream of the transcription initiation site of the main (P1) promoter and 210 bp of downstream 5' untranslated sequence from the ER promoter was linked with luciferase in the pGL2 vector (11). The work of Tang *et al.* have provided evidence suggested the presence of an enhancer element a bit upstream of this sequence, which they called ER-EH0 (12). We used PCR amplification from genomic DNA to amplify and then subclone into the promoter construct obtained from Dr. Weigel additional upstream sequence such that our full length (ER-3813-210Luc) ER promoter-luciferase construct (ER-3813-210Luc) now incorporates all known enhancer elements. This construct was used to prepare a panel of 5' deletion constructs by restriction enzyme digests, designed to sequentially remove the five documented sites reported to affect ER promoter transcription in various cell systems (Figure A).



**Figure A.** ER promoter luciferase constructs. The documented transcriptional elements are (a) ER-EH0 (12), (b) a negative regulatory element (13), (c) ERBF-1 (14), (d) ERUBF-1 (15), and (e) ERF-1 (11).

We had in our initial proposal planned to use the ER-positive cell line MCF7 for these experiments. However, due to a very high level of promoter activity even without co-transfection of a BRCA1 expression construct, and a high level of endogenous BRCA1 expression in MCF7 cells, we switched to use primarily a nontumorigenic ER-negative mammary epithelial cell line, MCF10A. MCF10A was chosen because of its ease of transfection (consistently greater than 50%), low endogenous level of BRCA1, and wild type *p53* status (16, 17). Previous investigation in our laboratory indicated that BRCA1 transactivation of the *p21* promoter is dependent on the *p53* status of the experimental cell line (16). In order to have another cell model with which to compare ER promoter results in MCF10A cells, we obtained a recently immortalized nontumorigenic mammary epithelial cell line, IMEC. IMEC cells were immortalized by initially introducing a recombinant retrovirus containing the gene for the catalytic subunit of human telomerase, *hTERT*, into primary human mammary epithelial cells (18).

The normalized luciferase values from these transfections are shown in Figure B. With MCF10A cells, we observed a nearly 50-fold increase from the full-length ER promoter construct, over the empty luciferase vector, when cotransfected with BRCA1, as compared with a 9-fold increase when cotransfected with the empty expression plasmid, pRK7. This induction was seen across seven

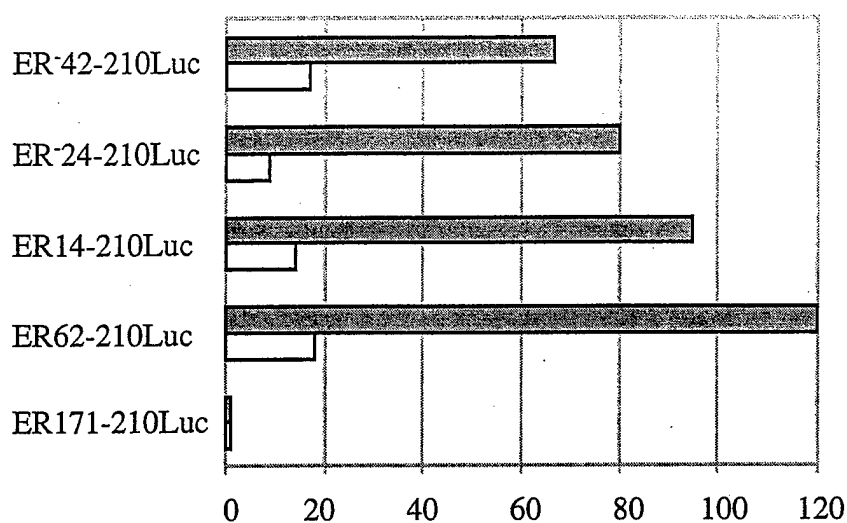


**Figure B.** Luciferase activity for ER promoter constructs in MCF10A and IMEC cells. Fold induction in luciferase activity (*x axis*) is shown for promoter constructs (*y axis*) following transfection with either a BRCA1 expression plasmid (*dark gray bars*) or the empty pRK7 vector (*white bars*). To calculate the fold induction in luciferase activity, the number of photon units per unit time of data capture (RLU) as read from a luminometer upon injection of luciferine, was divided by the amount of protein in whole cell extracts for each transfection plate. The RLU/protein value was further divided by the corresponding value from cells transfected with the empty pGL2 Basic vector. RLU-protein values from the empty pGL2 vector ranged from ~35 to ~400, while those from the ER promoter constructs started at ~1500. *X axis* values represent the fold induction in luciferase activity relative to the empty luciferase reporter vector following transfection with either BRCA1 or pRK7. Data shown represents the average of 2-7 experiments, with luciferase and protein measurements performed in triplicate for each experiment.

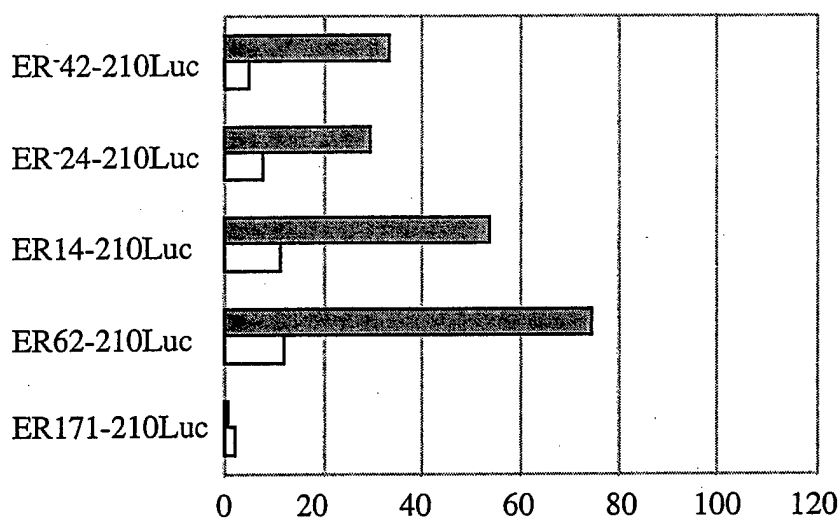
independent experiments. IMEC cells also showed a significant difference between BRCA1- and pRK7-induced luciferase activity with the full-length ER promoter construct: a 29-fold and 3.3-fold increase respectively ( $p < 0.0005$ ). Although the raw value of normalized luciferase activity varied across the deletion constructs in both MCF10A and IMEC cells, the induction of luciferase activity by BRCA1 as compared to pRK7 was not significantly different between any of these deletion constructs ( $p > 0.9$  in both MCF10A and IMEC cells). These results suggested that the ER promoter segment which mediates transactivation by BRCA1, was located within the region extending from 42 bp upstream to 210 bp downstream of the P1 transcriptional start site.

In order to localize more precisely the P1 region responsible for BRCA1 transactivation, we prepared additional deletion constructs of the ER promoter region by removing sequentially more sequence from the 5' end of ER42-210Luc. Since there were no unique restriction sites within the minimal ER42-210Luc construct with which to make the required deletion constructs, we utilized the unique properties of *ExoIII* nuclease to construct a series of unidirectional deletions of ER42-210Luc. Normalized luciferase activity of these constructs following cotransfection with either BRCA1 or pRK7 is shown in Figure C. Induction of promoter activity by BRCA1 did not significantly differ among ER24-210Luc, ER14-210Luc, and ER62-201Luc ( $p > 0.8$  and  $0.6$  for MCF10A and IMEC cells respectively). However as indicated in Figure C, the ER171-210Luc construct did not show any induction of luciferase activity upon transfection with either BRCA1 or pRK7. These data indicate that transactivation by BRCA1 is lost in ER171-210Luc, suggesting that the region mediating transactivation by BRCA1 is located between 62 bp and 171 bp downstream of the P1 transcriptional start site.

### MCF10A



### IMEC



**Figure C.** Luciferase activity for *ExoIII*-generated ER promoter constructs in MCF10A and IMEC cells. Fold induction in luciferase activity (*x axis*) is shown for ER promoter constructs following transfection with either a BRCA1 expression plasmid (*dark gray bars*) or the empty pRK7 vector (*white bars*). Fold induction values were calculated as in Figure B. Data shown represents the average of 2-7 experiments, with luciferase and protein measurements performed in triplicate for each experiment.

### Key Accomplishments

- Methodology for sequence-based analysis of methylation at 25 CpG within the ER promoter, following bisulfite modification of genomic DNA, has been developed and standardized with a panel of human breast cancer cell lines.
- Specimens of sporadic and *BRCA1*-linked ER-negative breast cancers have been analyzed at selected CpG sites within the ER-promoter, documenting an increased level of methylation within the *BRCA1*-linked tumors.
- An expanded ER promoter-luciferase construct, incorporating all known transcriptional enhancer elements for ER expression, as well as a series of 5' deletion constructs have been constructed.
- *BRCA1* has been shown to transactivate the ER promoter in two ER-negative immortalized mammary epithelial cell lines. The portion of the ER promoter mediating this effect of *BRCA1* has been localized to between 62 bp and 171 bp downstream of the main transcription start site.

### Reportable Outcomes

#### Publications:

William B. Archey, Kristen A. McEachern, Mark Robson, Kenneth Offit, Susan A. J. Vaziri, Graham Casey, Åke Borg, and Bradley A. Arrick. Increased CpG methylation of the estrogen receptor gene in *BRCA1*-linked estrogen receptor-negative breast cancers. *Oncogene*, in press (2002).

#### Manuscripts:

A manuscript is in preparation.

#### Abstracts:

William B. Archey, Kristen A. McEachern, Mark Robson, Kenneth Offit, Susan A. J. Vaziri, Graham Casey, Åke Borg, and Bradley A. Arrick. CpG methylation within the estrogen receptor promoter is increased in *BRCA1*-linked estrogen receptor-negative breast cancers. *Proc. AACR* 43:1114 (2002).

#### Patents, licenses, and inventions:

None

#### Degrees obtained:

Ph.D. June 2002 to Kristen McEachern (formerly Kristen Doherty)

Ph.D. June 2002 to William B. Archey

Both William Archey and Kristen Doherty, now Kristen McEachern, were supported by this award.

### Conclusions

The conclusion from the experiments for Task I is that ER-negative *BRCA1*-linked breast cancers are more highly methylated than ER-negative non *BRCA1*-linked tumors within the ER promoter region. This may represent one mechanism by which the ER gene is shut off in these tumors. Another

mechanism is highlighted by the data obtained in experiments for Task II. Namely, that BRCA1 can transactivate the ER promoter. Thus, in the absence of BRCA1, expression of the ER gene would be compromised at the level of transcription.

These conclusions have important implications with regard to furthering our understanding of the functions of *BRCA1* that are relevant to its role as a tumor suppressor gene in breast cancer. Furthermore, pharmacologic strategies for chemoprevention in mutation carriers are likely to be most effective if they account for (and counteract) the effects of BRCA1 loss in mammary epithelial cells. To the extent that alterations in epigenetic regulation (i.e. CpG methylation) underlie tumorigenesis in *BRCA1*-linked breast cancer, modifiers of CpG methylation may be of value.

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## ORIGINAL PAPERS

## Increased CpG methylation of the estrogen receptor gene in *BRCA1*-linked estrogen receptor-negative breast cancers

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A distinctive feature of *BRCA1*-linked breast cancers is that they typically do not express estrogen receptor- $\alpha$  (*ER $\alpha$* ). Previous investigation suggests that methylation of CpGs within the *ER $\alpha$*  promoter mediates repression of gene expression in some *ER $\alpha$* -negative breast cancers. To determine if methylation of CpGs within the *ER $\alpha$*  promoter is associated with *BRCA1*-linked breast cancers, we evaluated methylation in exon 1 of the *ER $\alpha$*  gene in 40 *ER $\alpha$* -negative breast cancers, 20 of which were non *BRCA1*-linked and 20 *BRCA1*-linked. CpG methylation was evaluated by either methylation-sensitive restriction digest (*HpaII*), methylation-sensitive PCR (MSP), or direct sequencing of bisulfite-treated genomic DNA. Results from *HpaII* digests and MSP documented a high degree of methylation, the MSP data showing slightly higher methylation in the *BRCA1*-linked group. CpGs analysed by direct sequencing showed an overall average methylation of 25% among non *BRCA1*-linked cancers and 40% among *BRCA1*-linked cancers ( $P=0.0031$ ). The most notable difference was found at five particular CpGs, each of which exhibited a greater than twofold increase in methylation in the *BRCA1*-linked group compared to the non *BRCA1*-linked group ( $P<0.03$  for each CpG). Methylation of certain critical CpGs may represent an important factor in transcriptional repression of the *ER $\alpha$*  gene in *BRCA1*-linked breast cancers.

*Oncogene* (2002) 00, 00–00. doi:10.1038/sj.onc.1205844

**Keywords:** *BRCA1*; estrogen receptor; methylation; breast cancer

### Introduction

*BRCA1* is a breast cancer susceptibility gene, germline mutations of which are linked to a significant

proportion of hereditary breast cancers (Miki *et al.*, 1994). Although the precise mechanisms by which mutations of *BRCA1* predispose the carrier to breast cancer are at present unknown, loss of the wild type allele appears to be a required event, thereby fulfilling the paradigm of a tumor suppressor gene (Neuhausen and Marshall, 1994; Cornelis *et al.*, 1995). The functions of the *BRCA1* protein that underlie its anti-oncogenic function are currently the subject of widespread investigation. *BRCA1* is indeed a multi-functional protein, implicated in three broadly defined cellular functions: transcriptional regulation, DNA repair, and cell cycle check point control (for a detailed review of the literature see Wang *et al.*, 2000; Zheng *et al.*, 2000; Welsh *et al.*, 2000). Nevertheless, studies thus far have yet to draw a conclusive association between any one of these functional roles and breast carcinogenesis. ②

Investigators have sought to identify characteristic phenotypic features of *BRCA1*-linked breast cancers. cDNA array assessments of mRNA expression profiles have suggested that these tumors exhibit distinctive patterns of gene expression (Hedenfalk *et al.*, 2001; Berns *et al.*, 2001; Van't Veer *et al.*, 2002). Immunohistochemical analyses have revealed that between two-thirds and 90% of *BRCA1*-linked breast cancers are *ER $\alpha$* -negative, making this one of the most distinguishing biological features of breast cancers that arise in carriers of a *BRCA1* mutation (Loman *et al.*, 1998; Jóhannsson *et al.*, 1997; Karp *et al.*, 1997). Recent data from Vaziri *et al.* (2001) suggest that the *ER $\alpha$* -negative phenotype is primarily a distinctive feature of *BRCA1*-linked breast cancers arising in women before menopause. Since one of the earliest molecular steps towards breast carcinogenesis is likely to be loss of the wild type copy of *BRCA1*, the predominance of *ER $\alpha$* -negativity in the subsequent cancers suggests that either the *ER $\alpha$* -negative phenotype has a selective advantage, or expression of *ER $\alpha$*  is compromised when *BRCA1* is absent. Considering the importance of estrogen-based signaling in the genesis and progression of breast cancer, this characteristic of *BRCA1*-deficient breast cancers has important consequences for treatment and prevention. As there is not a documented association

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between *BRCA1*-linked breast cancers and ER $\beta$ , this paper will only address the association with ER $\alpha$ .

ER $\alpha$ -negative breast cancers lack ER $\alpha$  mRNA, however the *ER $\alpha$*  gene is generally not mutated (Barrett-Lee et al., 1987; Piva et al., 1990; Yaich et al., 1992). Cytosine methylation within a dense cluster of CpG dinucleotides (i.e. CpG island) just downstream of the transcription initiation site of the P1 promoter of the *ER $\alpha$*  gene has been implicated as an epigenetic mechanism of transcriptional repression in ER $\alpha$ -negative cell lines and primary tumors. Ferguson et al. (1995) reported that inhibition of DNA methyltransferase in selected ER $\alpha$ -negative cell lines resulted in reexpression of the *ER $\alpha$*  gene. Lapidus et al. (1996) analysed DNA from ER $\alpha$ -negative primary breast cancers by Southern analysis with a methylation-sensitive restriction site, *NotI*, and detected methylation in 25% of the specimens. Later, this same group used MSP, a method by which bisulfite-treated DNA is amplified by primers specific for either methylated or unmethylated DNA, to document increased methylation at selected CpG sites in ER $\alpha$ -negative breast tumors as compared with ER $\alpha$ -positive tumors (Lapidus et al., 1998). Iwase et al. (1999) using PCR amplification across *HpaII* methylation-sensitive restriction sites as a measure of CpG methylation, more recently demonstrated methylation in ~80% of ER $\alpha$ -negative breast cancers. Of note, Iwase et al. (1999) also identified CpG methylation at *HpaII* sites in proximity to an upstream promoter (P0), but methylation at P0 was less clearly associated with the ER $\alpha$ -negative phenotype.

To determine if methylation of CpG dinucleotides within the P1 promoter of *ER $\alpha$*  is associated with *BRCA1*-linked breast cancers, we evaluated methylation at CpGs in exon 1 of the *ER $\alpha$*  gene, from -212 bps upstream to +240 bps downstream of the ATG start codon. Genomic DNA was prepared from formalin-fixed sections from 40 ER $\alpha$ -negative breast cancers, 20 of which were non *BRCA1*-linked and 20 *BRCA1*-linked. CpG sites were evaluated for methylation by one of the following three methods: *HpaII* digestion, MSP, or sequence analysis of bisulfite-treated genomic DNA. We demonstrate here a significantly higher level of CpG methylation in *BRCA1*-linked ER $\alpha$ -negative breast cancers compared with ER $\alpha$ -negative breast cancers that are not attributable to a germline mutation in *BRCA1*.

## Results

We began our investigation by analysis of cell line DNA using two independent established methods that previously have been employed to document methylation at specific CpG sites within the *ER $\alpha$*  promoter. The first method, PCR amplification of genomic DNA after restriction digest with the methylation-sensitive enzyme *HpaII*, does not require bisulfite treatment of the DNA. With this approach, Iwase et al. (1999) documented methylation at two CpG sites within exon

1 in ~80% of ER $\alpha$ -negative primary breast cancers. The second method we employed was MSP analysis of bisulfite-treated DNA. Of the six primer pairs tested by Lapidus et al. (1998) we selected ER1 and ER5. We chose the ER1 primer pair because the CpG situated at the 3' end of the lower ER1 primer is within the ERF-1 binding site, reported to play a role in ER $\alpha$  expression in some breast cancer cell lines (Deconinck et al., 1995). We chose the ER5 primer pair because MSP with these primers yielded the most significant difference in CpG methylation as a function of receptor expression in primary breast cancers (Lapidus et al., 1998).

Four ER $\alpha$ -negative cell lines were studied: MCF10A and 184B5 are both immortalized nontumorigenic mammary epithelial cell lines, MDA-MB-231 is a breast cancer cell line for which a large body of data regarding CpG methylation already exists, and HCC1937 is a recently developed cell line derived from a breast cancer arising in a woman with a germline mutation in *BRCA1* (Tomlinson et al., 1998). The left panel of Figure 1 shows PCR products of genomic DNA from each cell line after digestion with *HpaII*. By this assay, both the MCF10A and MDA-MB-231 cell lines showed notable methylation as evidenced by the presence of a PCR product in the *HpaII* digest lane, whereas the 184B5 and HCC1937 cells showed lack of methylation.

Results from MSP analysis of bisulfite-treated cell line DNA are illustrated in the right-hand panel of Figure 1. MCF10A cells showed the strongest overall methylation signal, especially within the ER1 region, while HCC1937 cells were totally unmethylated as indicated by lack of a PCR product with the methylated DNA-specific primers. Data from the *HpaII* digests most closely correlated with ER5 MSP data, as would be expected since one of the *HpaII* CpG sites is within the lower ER5 primer region while the other *HpaII* CpG is just 4 bps downstream of the lower ER5 primer.

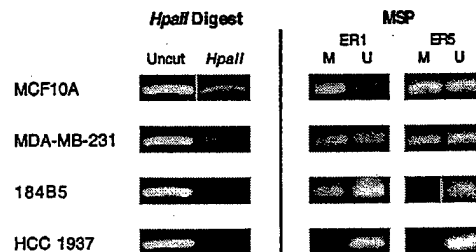


Figure 1 Methylation of CpG dinucleotides as determined by *HpaII* digests (left panel) and MSP (right panel) in four ER $\alpha$ -negative cell lines. Left panel, genomic DNA was digested overnight with *HpaII*, or left undigested (Uncut), then PCR amplified with primers that span the two *HpaII* sites. Presence of a PCR product in the *HpaII* column indicates methylation at both of the *HpaII* sites. Right panel, bisulfite-treated genomic DNA was PCR amplified with two sets of primer pairs (ER1 and ER5) specific for either methylated (M) or unmethylated (U) DNA

MSP analysis of DNA from three of the cell lines demonstrated the presence of both methylation and unmethylation at ER1 and/or ER5. This may reflect the fact that CpG methylation is often significantly less than 100%, as we have reported in an analysis of methylation at the TGF- $\beta$ 3 promoter (Archey *et al.*, 1999). Alternatively, this apparent heterogeneity within cell line DNA could have resulted from incomplete bisulfite-mediated conversion of unmethylated cytosines. To resolve this issue, and to provide data on the methylation status of significantly more than the handful of CpG sites queried by the above-utilized approaches, we performed sequence-based analysis of the bisulfite-treated DNA. The ER1 and ER5 regions were amplified from bisulfite-treated DNA with degenerate primers, such that both methylated and unmethylated template DNA would be co-amplified. The resulting PCR products were then directly sequenced, thereby providing data on 25 CpGs located within the amplified regions. Conversion of the non CpG cytosines was >95%, indicating that incomplete bisulfite treatment was not the reason for heterogeneity of methylation noted in the MSP analysis. Figure 2 shows the per cent methylation at each CpG site in our panel of ER $\alpha$ -negative cell lines. In support of the MSP data in Figure 1, MCF10A cells showed the highest level of methylation while HCC1937 cells showed the lowest level of methylation across the ER1 and ER5 regions. MDA-MB-231 cells, showing a heterogeneous MSP signal, were confirmed by sequencing to have an overall per cent methylation between that of MCF10A

cells and HCC1937 cells. 184B5 cells were shown by sequencing, as with MSP, to be highly methylated in ER1, but largely unmethylated in ER5 (Figures 1 and 2). As a measure of methylation across the ER1 and ER5 sequenced regions, we averaged the per cent methylation of all CpG sites. The average per cent methylation was 71% in MCF10A cells, 11% in HCC1937 cells, 39% in MDA-MB-231 cells and 32% in 184B5 cells. Of note, only the 184B5 cells demonstrated markedly different degrees of methylation between the ER1 and ER5 regions (56% in ER1 vs 1% in ER5).

Having established that comprehensive analysis of CpG methylation in this region of the ER $\alpha$  promoter, by three independent methods, could be performed with as little as 200 ng of cell line DNA, we turned our attention to analysis of DNA from a collection of ER $\alpha$ -negative primary tumor specimens, half of which were linked to BRCA1. Given that the HCC1937 cell line demonstrated minimal methylation throughout this region, we first analysed DNA isolated from patient specimens to test the hypothesis that BRCA1-linked tumors would show significantly less methylation than the non-linked specimens. For this purpose, we applied a method with perhaps the highest sensitivity for the presence of CpG methylation: PCR amplification following digestion of DNA with a restriction enzyme that will leave uncut templates with methylated CpGs at the restriction site. As previously discussed, using this methodology, Iwase *et al.* (1999) reported that ~80% of ER $\alpha$ -negative breast cancers from women

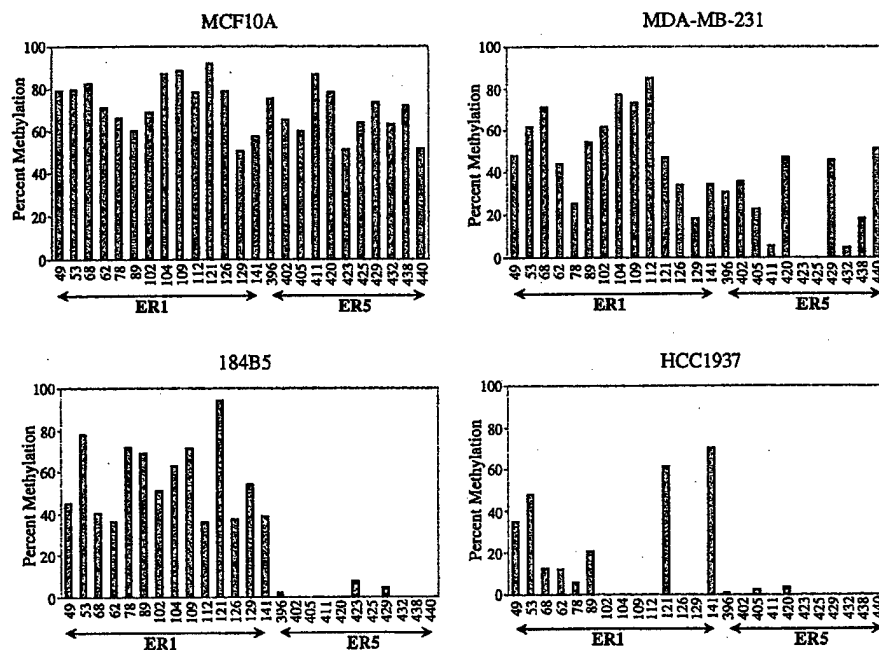


Figure 2 CpG methylation of the ER $\alpha$  promoter in four ER $\alpha$ -negative cell lines. Bisulfite-treated DNA from each cell line was amplified with degenerate primers for the ER1 and ER5 regions, and then sequenced as described in Materials and methods. CpG location is indicated (x axis) relative to the first nucleotide of P1. MCF10A cells, MDA-MB-231 cells, 184B5 cells, and HCC1937 cells are shown

without a positive family history showed methylation at two CpG sites within a *HpaII* site in proximity to the ER5 downstream primer. Analysis of *HpaII* digested DNA from the patient specimens demonstrated significant methylation (94%) within the *BRCA1*-linked specimens (data not shown). Among the non-*BRCA1*-linked group 81% showed methylation, consistent with the published data of Iwase *et al.* (1999). While the difference between the *BRCA1*-linked and non-*BRCA1*-linked groups was not statistically significant ( $P > 0.2$ ), these data negate the hypothesis that *BRCA1*-linked breast cancers are largely unmethylated at the ER $\alpha$  locus, as was the case with the HCC1937 cell line, and that in this context the HCC1937 cell line is not representative of primary *BRCA1*-linked breast cancers.

As a semi-quantitative measure of the relative abundance of methylated and unmethylated DNA at the ER1 primer binding sites, we amplified bisulfite-treated DNA with the methylated DNA-specific primer pair as well as with the degenerate primer pair in parallel amplification reactions and compared the relative intensity of the resulting PCR products on an agarose gel (data not shown). In separate reactions utilizing synthesized templates representing methylated and unmethylated ER1 sequence, we determined that PCR products of equal intensity with these primer pairs resulted when the methylated DNA template constituted ~10% of the total, reflecting a lower efficiency of amplification with the degenerate primers (not shown). We observed that whereas only one out of 12 samples in the non-*BRCA1*-linked group produced a PCR band of greater intensity with the methylated DNA-specific primers than with the degenerate primers, half of the *BRCA1*-linked group (four out of eight) yielded a PCR band of greater intensity with the methylated DNA-specific primers than with the degenerate primers. Although this represents a significant difference between the two groups ( $P = 0.035$  by *Chi-Square* analysis), the limited number of both specimens and CpG sites evaluated diminished the interpretive power of these data. We therefore decided to devote the limited amount of DNA from the tumor specimens available to the more comprehensive sequence-based analysis of bisulfite-treated DNA in order to test the hypothesis that CpG methylation at the ER $\alpha$  promoter is increased in ER $\alpha$ -negative *BRCA1*-linked breast cancers.

Bisulfite-treated DNA from the tumor specimens was amplified with degenerate primers for both the ER1 and ER5 regions, and then analysed by DNA sequencing to ascertain the per cent methylation at each of 25 CpG sites (see Materials and methods for a full description of this assay). Figure 3 illustrates the average methylation at each CpG for the non-*BRCA1*-linked and *BRCA1*-linked breast cancer groups. Methylation was higher at most CpG sites in the *BRCA1*-linked breast cancers as compared to the non-*BRCA1*-linked breast cancers in both the ER1 and ER5 regions (Panels A and B of Figure 3, respectively). The overall average per cent methylation was 25%

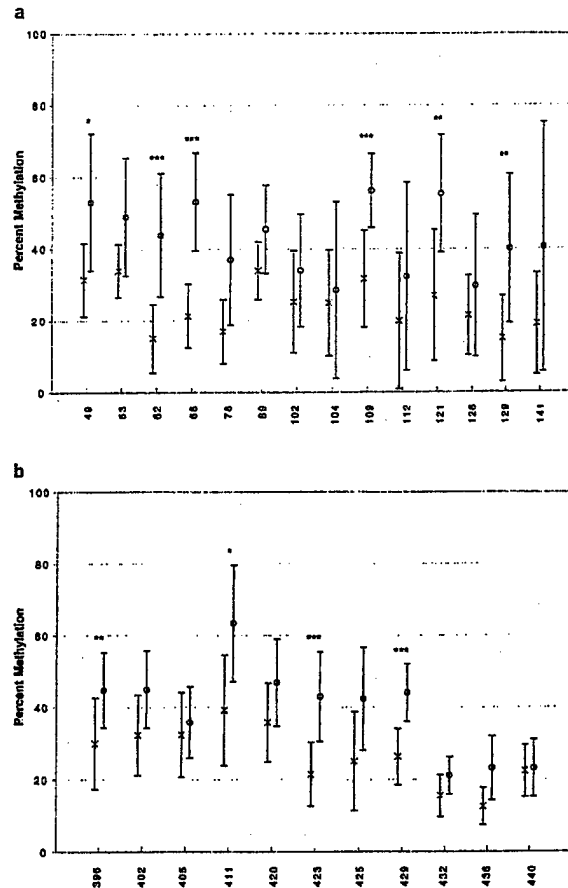
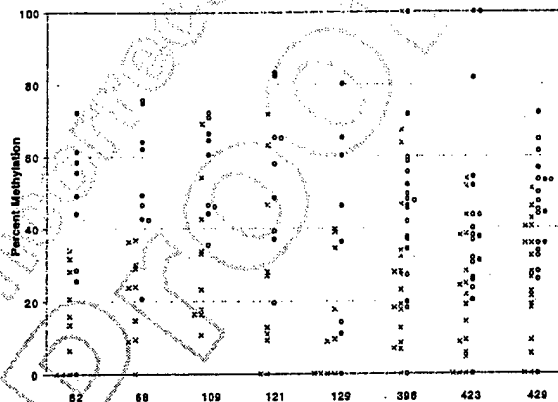


Figure 3 CpG methylation of the ER $\alpha$  promoter in primary breast cancer samples. Bisulfite-treated DNA from each primary section was amplified with degenerate primers for the ER1 (Panel A) and ER5 (Panel B) regions, and then sequenced as described in Materials and methods. The average per cent methylation,  $\pm 95\%$  confidence interval, for non-*BRCA1*-linked breast cancers (X) and *BRCA1*-linked breast cancers (O) is indicated for each CpG site. Significant differences between the two groups are illustrated as  $P < 0.05$  (\*),  $P < 0.03$  (\*\*) and  $P < 0.01$  (\*\*\*)

among non-*BRCA1*-linked cancers and 40% among *BRCA1*-linked cancers ( $P = 0.0031$ ). Specifically within the ER1 regions, the overall average per cent methylation was 24 and 43% ( $P = 0.0041$ ) respectively, and within ER5, 27 and 39% ( $P = 0.0094$ ) respectively. There were no significant differences in the overall average per cent methylation between the ER1 and ER5 regions in the primary specimens. Of note, five of the 25 examined CpGs, located at positions 62, 68, 121, 129 (Panel A), and 423 (Panel B), exhibited a greater than twofold increase in average methylation in the *BRCA1*-linked group compared to the non-*BRCA1*-linked group ( $P < 0.03$ ).

Of the 25 CpG sites investigated by sequencing, eight demonstrated significantly more methylation in the *BRCA1*-linked specimens compared with the non-*BRCA1*-linked specimens ( $P < 0.03$  for each CpG), five of which demonstrated no overlap in the 95%



① Figure 4 Distribution of methylation at significant CpG sites ( $P < 0.03$ ) as indicated by individual primary breast cancer sections. Per cent methylation for each non *BRCA1*-linked breast cancer specimen (X) and *BRCA1*-linked breast cancer specimen (O) is shown at the eight most discriminative CpG sites. Methylation within the *BRCA1*-linked group is significantly higher than in the non *BRCA1*-linked group at each CpG site ( $P$  values from left to right are 0.0076, 0.0006, 0.0057, 0.022, 0.0279, 0.0218, 0.0082 and 0.002)

confidence intervals ( $P < 0.01$  for each CpG). Data from the individual specimens for the panel of eight discriminative CpG sites are presented in Figure 4. It is evident from examination of the individual data points that clear outliers do exist for both the non-*BRCA1*-linked and *BRCA1*-linked groups.

## Discussion

Perhaps the most distinctive pathologic feature of breast cancers that arise in women who inherit a mutation in *BRCA1* is a lack of expression of  $ER\alpha$ . Mechanisms associated with the  $ER\alpha$ -negative phenotype in breast cancer cell lines include absence of requisite transcription factors, presence of DNA-binding factors that repress transcription, and epigenetic changes such as DNA methylation and histone deacetylation (Ferguson et al., 1995; Tang et al., 1997; Mcpherson et al., 1997; Penolazzi et al., 2000; Yang et al., 2000). Analysis of DNA from primary breast tumors has revealed methylation of CpGs within exon 1 of the *ER\alpha* gene in a significant portion of  $ER\alpha$ -negative cancers, but rarely in  $ER\alpha$ -positive tumors. The present study was done to determine whether the  $ER\alpha$ -negative phenotype among *BRCA1*-linked breast cancers was similarly associated with CpG methylation. We observed that  $ER\alpha$ -negative breast cancers arising in *BRCA1* mutation carriers were even more extensively methylated than  $ER\alpha$ -negative cancers from women without a *BRCA1* mutation. A potential weakness in our analysis is that microdissection of the tumor cells away from normal cells, which would contribute DNA that is unmethylated at this locus, was not performed.

Approximately 10% of *BRCA1*-linked specimens showed low levels of methylation (Figure 4). Thus, even within this relatively homogeneous collection of tumors, there was evident heterogeneity in methylation. Clearly, not all  $ER\alpha$ -negative breast cancers that develop in *BRCA1* mutation carriers arise via the same sequence of cancer-promoting cellular changes. Noting that the HCC1937 cell line was also minimally methylated in this region, it seems likely that this line originated from a breast cancer that is representative of the ~10% of *BRCA1*-linked  $ER\alpha$ -negative tumors lacking hypermethylation in this region. Although not studied here, we would expect CpG methylation to be absent in the 10–30% of *BRCA1*-linked breast cancers that do express  $ER\alpha$ .

From our sequence-based analysis of multiple CpG sites, we observed that the degree of methylation differed somewhat from one CpG to another, which might explain why different methods that are based on only a few CpG sites could generate different estimates of the frequency of methylation. Furthermore, although an overall higher level of methylation in the *BRCA1*-linked specimens was found throughout the analysed portions of the *ER\alpha* gene, a subset of the CpG sites provided the greatest distinction between *BRCA1*-linked and non-*BRCA1*-linked tumors. We hypothesize that methylation of these most discriminating CpGs is integral to the mechanism by which loss of *BRCA1* function results in the lack of  $ER\alpha$  expression.

A correlation between loss of *BRCA1* and the  $ER\alpha$ -negative phenotype may extend to a substantial portion of sporadic breast cancers as well. In recent years various investigators have reported that approximately 20–35% of sporadic invasive ductal carcinomas expressed very low or undetectable levels of *BRCA1* protein (Wilson et al., 1999; Yoshikawa et al., 1999). Furthermore, sporadic breast cancers that lack *BRCA1* expression, in some instances due to CpG methylation within the *BRCA1* promoter, may be more likely to be  $ER\alpha$ -negative (Catteau et al., 1999). From the data in Figure 4 it is evident that approximately 25–40% of the  $ER\alpha$ -negative non-*BRCA1*-linked specimens showed a high degree of CpG methylation, to the level that was characteristic of the *BRCA1*-linked group. We therefore hypothesize that decreased expression of *BRCA1* in a subset of sporadic breast cancers results in loss of  $ER\alpha$  expression via CpG methylation. Correlation of *BRCA1* expression with *ER\alpha* methylation status in a collection of sporadic cancers would serve to test this hypothesis.

There are no available data concerning a direct role of *BRCA1* regarding the expression or function of any of the known DNA methyltransferases, or demethylase enzymes. *BRCA1* has been reported to physically associate with components of the histone deacetylase complex, although the functional consequences of these interactions are unclear (Yarden and Brody, 1999). An evolving notion in the literature is that a dynamic interplay between histone acetylation and CpG methylation, whereby each influences the other, acts as a mechanism to magnify and perpetuate epigenetic

control of gene expression (Dobosy and Selker, 2001; Cervoni and Szyf, 2001). Considering the large number of genes whose expression level has been reported to differ as a function of BRCA1 expression, it is intriguing to suggest that BRCA1 influences an important epigenetic network that affects the expression of multiple genes.

In summary, we have observed that CpG methylation in ER $\alpha$ -negative breast cancers is significantly more extensive among BRCA1-linked tumors. If expression of ER $\alpha$  within mammary epithelial cells lacking BRCA1 can be induced by compounds that inhibit DNA methylation or histone deacetylation, the clinical development of such agents may be of particular use to reverse some of the epigenetic consequences of the loss of BRCA1 in breast cancer.

### Materials and methods

#### Cell culture

Cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). MCF10A and MDA-MB-231 cells were cultured in DMEM:Ham's F-12 medium, supplemented with 100 IU/ml of penicillin, 125  $\mu$ g/ml of streptomycin, 2 mM L-glutamine and 10% fetal bovine serum. MCF10A culture medium was additionally supplemented with 20 ng/ml of epidermal growth factor, 0.5  $\mu$ g/ml of hydrocortisone and 8  $\mu$ g/ml of insulin. 184B5 cells were cultured in MEMB medium (Clonetics, Walkersville, MD, USA), supplemented with penicillin, streptomycin and L-glutamine as above, in addition to 5 ng/ml of epidermal growth factor, 0.5  $\mu$ g/ml of hydrocortisone, 5  $\mu$ g/ml of insulin, 5  $\mu$ g/ml of transferrin and 10<sup>-5</sup> M isoproterenol. HCC1937 cells were cultured in RPMI 1640 medium with L-glutamine, supplemented with 125  $\mu$ g/ml streptomycin and 10% fetal bovine serum.

#### Patient specimens

Paraffin-embedded, formalin-fixed specimens that met the following criteria were obtained from collaborating institutions. At the originating institution, tumors were determined to be ER $\alpha$ -negative by immunohistochemistry and were histologically classified as either infiltrating ductal or medullary. The BRCA1-linked specimens came from women with a pathogenic germline BRCA1 mutation. The non-BRCA1-linked specimens came from women who either had no first degree relative with breast or ovarian cancer, and therefore had a negligible *a priori* risk of being a BRCA1 mutation carrier, or had undergone genetic testing and did not carry a detectable BRCA1 mutation. The average age at diagnosis for the BRCA1-linked group (43  $\pm$  12 years) did not differ significantly from the average age at diagnosis for the non BRCA1-linked group (50  $\pm$  11 yrs).

#### DNA isolation

Cell line genomic DNA was isolated from adherent cells using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA). For preparation of DNA from breast cancer specimens, 10–20 micron sections were first deparaffinized with Stephens Clearing Solvent (Stephens Scientific, Riverdale, NJ, USA), washed twice in 100% ethanol and dried at 37°C in a sandbox. Digestion of tissue

and isolation of genomic DNA was performed using the Qiagen DNA Mini Kit (Qiagen, Valencia, CA, USA). Tissue digestion was performed overnight at 56°C with 12–36 mAU proteinase K, and genomic DNA was eluted in 300  $\mu$ l H<sub>2</sub>O. One-half of each sample was apportioned to analysis of non bisulfite-treated DNA, i.e. HpaII digest, while the other half was apportioned to analysis of bisulfite-treated DNA, i.e. MSP and sequencing of genomic DNA.

#### HpaII digests

A modified procedure from Iwase *et al.* (1999) was used for HpaII digests. In brief, one hundred nanograms of cell line DNA, or alternatively one-sixth of the total DNA isolated from a given primary tumor sample, was subjected to restriction digest with 50 U of HpaII (NE Biolabs, Beverly, MA, USA) or no enzyme in 0.5  $\times$  Universal Buffer (Stratagene, La Jolla, CA, USA) in a final volume of 30  $\mu$ l. Digestion took place for 20 h at 37°C. One third of the digestion reaction was used for PCR amplification of a 230 bp region using the following primers: upper 5'-AGCAGCAAGCCCGCCGTGTACAAC-3', lower 5'-GGGCTGCAGGAAAGGCGACAGC-3'. Each PCR reaction contained 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.5  $\mu$ M each primer, and 2.5 U Taq Platinum (Gibco, Gaithersburg, MD, USA) in a total volume of 60  $\mu$ l with 20 mM Tris-HCl (pH 8.8). PCR reaction conditions were as follows: initial soak of one cycle at 95°C for 5 min, followed by 38 cycles at 94°C for 30 s, 67°C for 30 s and 72°C for 30 s, followed by extension for one cycle at 72°C for 10 min. One half of the total PCR volume was resolved on a 1.2% TBE-agarose gel, stained with ethidium bromide and visualized by UV transillumination.

#### Bisulfite modification of DNA

Genomic DNA apportioned to MSP and sequencing analysis was supplemented with 1  $\mu$ g of salmon sperm DNA as a carrier, and denatured in a volume of 50  $\mu$ l in the presence of 0.2 M NaOH at 37°C for 10 min. As outlined by Herman *et al.* (1996), denatured DNA was treated with sodium bisulfite, desalted using the Wizard DNA Clean-Up System (Promega, Madison, WI, USA) and desulphonated with 0.3 M NaOH at room temperature for 5 min. DNA was then precipitated in EtOH and resuspended in TE buffer for storage at -80°C.

#### MSP and amplification of bisulfite-treated DNA with degenerate primers

Approximately one-tenth of the total DNA isolated from a given primary tumor sample, or 30 ng of cell line DNA, was used as template for PCR after bisulfite treatment. A modified procedure of Lapidus *et al.* (1998) was used for PCR amplification using the primer sets designated as ER1 and ER5. In brief, each PCR reaction contained 1  $\times$  PCR buffer prepared fresh (16.6 mM ammonium sulfate, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl<sub>2</sub> and 10 mM  $\beta$ -mercaptoethanol), 1.25 mM each dNTP, 150 ng each primer, and DNA template in a final volume of 25  $\mu$ l. Reactions with ER1 primers contained 0.72 U Taq Platinum and reactions with ER5 primers contained 0.63 U Taq Platinum. ER1 degenerate primer sequences were as follows: upper 5'-TTTTGGGATTGTATTTGTTTTYGYG-3', and lower 5'-AACAAAATACAAACCRATCCCCR-3'. These primers correspond to nucleotides 21–45, and 193–217, relative to the first nucleotide of transcription from P1. ER5 degenerate primer sequences were as follows: upper 5'-GTGATTTG-

GATAGTAGTAAGTTYGTY-3', and lower 5'-CRTAAAA-AAAACCRATCTAACCR-3'. These primers correspond to nucleotides 355-381, and 450-472, relative to P1. Degenerate primers were characterized by 'Y' or 'R' sites shown in bold, which designate a 50/50 mixture of cytosines and thymines (Y), or guanines and adenines (R). Each PCR reaction underwent initial denaturation at 95°C for 5 min, followed by either 42 cycles (ER1) or 40 cycles (ER5) of the following profile: 30 s at 94°C, 30 s at 58°C (ER1) or 30 s at 55°C (ER5), and 30 s at 72°C. Each reaction completed its PCR cycle profile with a 10 min extension at 72°C. For each primary breast cancer sample, three PCR reactions were performed: ER1 with methylated-DNA specific primers, ER1 with degenerate primers, and ER5 with degenerate primers. Seventeen microliters of each 25 µl reaction was resolved on a 2% TBE-agarose gel, stained with ethidium bromide and visualized by UV transillumination.

#### Sequencing of PCR reaction products

PCR products from bisulfite-treated DNA using the degenerate primers were reamplified using the same degenerate primers and PCR conditions, and 5 µl of the previous reaction as a template. Reamplified PCR products were purified using the Qiagen PCR Clean Up Kit (Qiagen, Valencia, CA, USA) and sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing System (Perkin Elmer Corp., Foster City, CA, USA). ER1 and ER5 degenerate primers were used for sequence via primer extension with the following conditions: 10 s at 96°C, 5 s at 50°C and 4 min at 60°C for 25 cycles. Sequencing reaction solutions were purified using Centriflex Gel Filtration Cartridges (Advanced Genetic Technologies, Gaithersburg, MD, USA). Percent methylation levels were calculated from the fluorescence intensities at the CpG sites of interest. The levels of cytosine (the presence of which indicates methylation as sodium bisulfite did not convert it) and thymine (the presence of which indicates unmethylation as sodium bisulfite did convert it) fluorescence peak heights were used as a reflection of the

fraction of DNA in the sample that was methylated at that position, since nucleotide fluorescence peak heights can vary in intensity depending on their location within a given sequencing region, peak height ratios were normalized using a standard curve based upon sequencing reactions with defined mixtures of plasmid DNA containing ER1 and ER5 DNA sequence representative of methylated and unmethylated DNA after bisulfite treatment. Oligonucleotides were designed and synthesized to reflect bisulfite-converted methylated and unmethylated DNA, amplified with the ER1 and ER5 primers, and subcloned into a pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA). Column-purified plasmid preparations incorporating sequences representative of either fully methylated or unmethylated DNA were combined (mixtures containing 10, 25, 33 and 50% of DNA sequence reflective of CpG methylation) and sequenced, and a standard curve was determined for each CpG position using a linear regression formula. The known fraction of methylated DNA was plotted against the cytosine to thymine peak height ratios taken from the sequencing electropherograms to establish the standard curve, against which per cent methylation at each CpG site in each tumor specimen was determined.

#### Statistical analyses

Significance of the per cent methylation among primary breast cancer samples in Figures 3 and 4 was calculated using a two-tailed, nonparametric Mann-Whitney test suitable to data sets with skewed distributions.

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