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PRINCIPAL INVESTIGATOR: Kristen A. Doherty

CONTRACTING ORGANIZATION: Dartmouth College
Hanover, New Hampshire 03755-1404

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13. ABSTRACT (Maximum 200 Words) The overall goal of our research is to investigate the correlation between BRCA1-mutated breast cancers and the Estrogen Receptor (ER)-negative phenotype. At this point in our work, experiments with patient specimens are too preliminary for conclusions. We have analyzed DNA from a panel of cell lines as we've worked out the specific conditions of our assays, as well as to allow for correlation with published literature with these cell lines. These analyses have revealed heterogeneity in the CpG methylation status of the ER promoter in ER-negative cell lines that had not previously been appreciated. Whereas some such cell lines show methylation at this promoter (e.g. MDA-231 cells), as reported in the literature, others do not. For instance, minimal-to-no methylation was evident in the HCC1937 breast cancer cell line (derived from a BRCA1 mutation carrier) or the 184B5 line of immortalized mammary epithelial cells. Neither of these lines have been included in prior reports of ER promoter methylation in the past. It is as yet unclear which, if any, of these cell lines will be representative of the status found from actual tumor specimens.				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4-6
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	7
References.....	7-8
Appendices.....	

Overview:

The overall goal of our research is 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-associated 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). Methylation of CpGs within the ER promoter has been implicated as an operative mechanism of repressed expression in some cell lines and tumor specimens. In accordance with the Statement of Work, all of this first year of funding was devoted to our first aim: to compare the methylation status of selected CpG sites located in the ER promoter region between ER-negative BRCA1-associated breast cancers and BRCA1-wildtype ER-negative tumors.

Progress to Date:

In our original proposal, we outlined as our intended approach to the analysis of CpG methylation at the ER promoter a plan to first treat the DNA with sodium bisulfite and then amplify a 230 bp fragment that would include the ATG site, a BstUI site just upstream of the start site, and the ERF-1 binding site described by DeConinck et al. (9). The BstUI site coincides with a SacII site that had been reported by Ottaviano et al. to be methylated in ER-negative cell lines (10). Our plan was to adapt the COBRA assay, which is based on the methylation status of the two CpGs within the BstUI site (11). In preliminary experiments with cell line DNA, we found that amplification of this region from bisulfite-treated DNA was erratic. Furthermore, sequencing of bisulfite-treated DNA from an ER-negative cell line reported by others to be heavily methylated at CpGs in this region revealed that only one of the BstUI CpGs was methylated. Since the COBRA assay will only indicate a site as 'methylated' if both CpGs are methylated, it became clear that the COBRA assay would be unable to identify those specimens with CpG methylation that is associated with the ER-negative phenotype.

As an alternative approach, we turned to an assay published by the Davidson group (John Hopkins) to evaluate the CpG status of the ER promoter in tumor specimens utilizing so-called Methylation Specific PCR (MSP) of bisulfite-treated DNA (12). This method is based on the design 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. For our work, we selected primer pairs ER1 and ER5. We chose ER1 because one of the CpGs this primer pair queries is the CpG within the ERF-1 binding site (9). We chose the ER5 primer pair because results with this

pair resulted in the greatest distinction between ER-positive and ER-negative tumors, with ER-negative tumors demonstrating significantly more methylation than ER-positive tumors (~80% methylation in the ER-negative group) (12).

We first established the performance of this assay of ER promoter methylation with a panel of six human breast cell lines, including the HCC1937 BRCA1-null cancer cell line. Our results are depicted in Table 1. Methylation was not evident in the ER-positive line (MCF-7), but was readily apparent in three of the five ER-negative lines (MDA-231, MCF-10A, and Hs578t). Of note, the cells from the BRCA1 mutation carrier displayed no methylation at these sites, and thus was in this manner indistinguishable from the ER-positive MCF-7 cells.

Table 1. ER Promoter CpG Methylation Results

Cell lines (ER status)	<i>ER1</i>	<i>ER1</i>	<i>ER5</i>	<i>ER5</i>
	<i>Methylated</i>	<i>Unmethylated</i>	<i>Methylated</i>	<i>Unmethylated</i>
MCF-7 (ER+)	–	+	–	+
MDA-231 (ER-)	- / +	+	+	+
MCF-10A (ER-)	+	–	+	+
184B5 (ER-)	- / +	+	–	+
HCC1937 (ER-)	–	+	–	+
Hs578t (ER-)	+	+	+	+

Genomic DNA from the indicated lines (100 ng, mixed with 1 µg salmon sperm DNA) was denatured and treated with sodium bisulfite, as per (13), then apportioned for separate PCR amplification with ER1 methylation primers, ER1 unmethylated primers, ER5 methylated primers, or the ER5 unmethylated primer pair, as in (12). The + or – in the table indicates whether a PCR product is obtained. Negative controls were included in each PCR reaction. The presence of a PCR product with at least one of the primer pairs for each cell line serves as a control for the presence of amplifiable DNA after the bisulfite treatment. For both MDA-231 and 184B5 cells, in one experiment no product was obtained with the ER1 methylation-specific primers whereas in another experiment PCR products were obtained.

As outlined in our proposal, our intention is to apply these molecular analyses to DNA recovered from formalin-fixed paraffin-embedded sections of breast tumor specimens from patients. We will compare ER-negative tumors from patients unlikely to have a BRCA1 germline mutation (i.e. lacking a family

history and therefore likely a sporadic breast cancer case) with ER-negative tumors from patients with known germline BRCA1 mutations. We have begun to work with the patient specimens, and have preliminary data. With the ER1 primers, with seven sporadic breast cancer specimens all were found to yield low levels of a methylated product (compared with PCR with degenerate PCR primers). In contrast, analysis of 5 BRCA1-derived specimens demonstrated high levels of methylation in 80% (4 out of 5). We found that we recovered too little DNA for the analogous approach with the ER5 primers, and so we resorted to an alternative plan: amplification of bisulfite-treated DNA with degenerate ER5 primers (which don't distinguish between methylated and unmethylated templates), followed by sequencing. By this approach, we are able to ascertain the methylation status of 11 CpG sites within the amplified segment of DNA. When DNA from the HCC1937 or 184B5 cells was used, the CpG sites were found to be predominantly unmethylated, whereas when starting with DNA from the MCF-10A cells a mixture of methylated and unmethylated sequences were observed at these CpG sites. We are now beginning to apply this method to our patient-derived specimens. As seen in the ER1 region, the BRCA1-derived samples exhibit more methylation than the sporadic specimens.

Key Accomplishments

- Methodology for analysis of CpG methylation at the ER promoter that is applicable to DNA recovered from formalin-fixed patient specimens has been developed and standardized with a panel of human breast cancer cell lines
- Specimens of sporadic and BRCA1-derived ER-negative breast cancers have been collected.
- We have begun to analyze the specimens.

Reportable Outcomes

none

Training Activities

In this past year, the supported student attended the annual meeting of the American Association for Cancer Research (in San Francisco). In addition, she took a course at Dartmouth in Epidemiology (grade: Honors).

Conclusions

Experiments with patient specimens are too preliminary for conclusions. The analysis of cell line DNA has revealed heterogeneity in the CpG methylation status of the ER promoter in ER-negative cell lines that had not previously been appreciated. Whereas some such cell lines show methylation at this promoter, as reported in the literature, others do not. For instance, minimal-to-no methylation was evident in the HCC1937 breast cancer cell line or the 184B5 line of immortalized mammary epithelial cells. Neither of these lines have been included in prior reports of ER promoter methylation in the past. It is as yet unclear which, if any, of these cell lines will be representative of the status found from actual tumor specimens.

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