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Methylation of ESRα Promoters in Benign Breast Tumors Could Be a Signature for Progression to Breast Cancer in African American Women

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Abstract

Background/Aim: Methylation in the estrogen receptor alpha (ESRα) promoter is an epigenetic abnormality associated with breast cancer (BCa), whereas hypermethylation results in the loss of ER expression.

Materials and Methods: Pyrosequencing was used to investigate a potential link between aberrant methylation in the P0/P1 promoters of ESRα and the risk of progression of benign fibrocystic and fibroadenoma tumors to BCa.

Results: Results showed a significantly elevated level of DNA methylation in ESRα P1 promoter (p=0.0001) in fibroadenoma compared to ER-negative BCa tumors and a two-fold increased ESRα expression in fibrocystic and fibroadenoma benign tissues. In addition, methylation levels of HIN-1 and RASSF1A promoters were elevated in ER-positive compared to ER-negative BCa (p-value<0.04). ANOVA Mixed Model revealed significantly higher methylation levels in the promoter of RASSF1A for fibroadenoma and ER-positive BCa (p=0.004) compared to ER-negative BCa. Tumors with unclassified molecular subtypes (ER-positive, PR-negative, HER2-negative) had elevated levels of methylation (p=0.046) in the P0 promoter compared with luminal B (ER-positive, PR-positive, HER2-positive) tumors. Grade 3 tumors showed a borderline association with ESRα P1 promoter methylation when compared with grade 2 tumors (p=0.056).

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Conclusion: ESRα P0 promoter hypermethylation may occur in the early stages of breast carcinogenesis, while P1 promoter methylation appears in later stages with a poor prognosis. Therefore, methylation of the ESRα promoter and other tumor-related genes could serve as a potential biomarker for predicting fibroadenoma progression risk to BCa.

Keywords: DNA methylation, estrogen receptor alpha, breast cancer, fibroadenoma.

sIntroduction

Breast cancer (BCa) is one of the most common malignancies in women, and the incidence and distribution vary widely among women of different ethnic backgrounds (1). The prevalence of BCa is slightly higher for white women, but the mortality rate for African American women is significantly higher (2).

BCa is a typical hormone-dependent tumor and has been sub-categorized based on the presence or absence of estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) (3). ER is a member of a large family of nuclear transcription regulators that function as nuclear transcription factors and co-regulators to enhance estrogen/non-estrogen target genes. The two isoforms of ER are alpha (α) and beta (β), and both are expressed in the normal mammary gland (4). *ESRα* is critical for normal mammary gland ductal development (5-7). *ESRβ,* however, is considered a putative tumor suppressor gene (8, 9). Both ER and PR are expressed in benign fibrocystic lesions (10), whereas fibroadenomas almost universally express PR, while ER is only expressed in approximately one-fourth of these benign tumors (11). In the earliest stages of BCa progression, the expression of *ESRα* increases, leading to ductal hyperplasia (DH), and the relationship between lack of *ESRα* expression and lack of proliferation of ESRα-positive cells changes (12). If *ESRα* expression continues to increase, tissues progress to atypical ductal hyperplasia (ADH) and low-grade ductal carcinoma in situ (DCIS) (13, 14). During these steps, lesions can lose their *ESRα* expression as carcinoma in situ progresses to invasive carcinoma (15, 16). DNA methylation and chromatin remodeling are two epigenetic mechanisms linked with the lack of *ESRα* expression in ESRα-negative BCa (17-20). Methylation is catalyzed by enzymes called DNA methyltransferases (DNMTs) that transfer a methyl moiety from S-adenosylmethionine to the 5´ positions of the cytidine ring to form 5-methyl-cytosine (21). In mammals, cytidines, usually followed by guanosine (CpGs), are methylated. CpGs are locally enriched in short stretches of DNA called CpG islands and are found in approximately 40% of mammalian promoters (22-24).

The methylation of *ESRα* has been well studied and is focused on the 5' regulatory region, which encompasses the promoter and the first exon (25). Methylation of CpG islands is correlated with transcription silencing of *ESRα* through direct gene silencing and formation of heterochromatin (26). *ESRα* mRNA is primarily transcribed from two promoters, the distal (P0) and the proximal (P1). The P1 promoter transcript is the major transcript in breast tissues, while the P0 promoter transcript is involved in tissue-specific developmental regulation of *ESRα*. The two transcripts share identical sequences encoding the same ER protein but differ in upstream regions. The P1 promoter is located upstream near the major transcription mRNA1 cap site, and the P0 promoter is located upstream of nucleotide -3067 relative to the P1 promoter (27). The transcribed messages from both promoters are almost identical isoforms due to alternative splicing. The only difference between the two transcripts is in the 5' region, which is unique for each transcript (28). However, Hayashi *et al.* (29) reported a positive correlation between breast tumor ER-protein expression and relative usage of the distal promoter in

post-menopausal women, a correlation not observed in pre-menopausal women.

In cancerous tissues, silencing of genes through promoter methylation occurs as frequently as mutations or deletions (30). Lack of *ESRα* expression in ERα-negative (ER-negative) BCa is associated with an increase in DNMTs and methylation of *ESRα* promoter (31, 32). Promoter hypermethylation is also a contributing factor in the early stages of carcinogenesis, and hypermethylation-mediated loss of gene expression could provide the cell with growth-promoting characteristics, such as insensitivity to antigrowth signals, limitless replicative potential and evasion of apoptosis (30, 33-36). Porras and colleagues (37) reported that ESRα is expressed in a subset of luminal cells, corresponding to less than 10% of normal mammary epithelial cells and over 70% of breast tumors (ER-positive BCa). Still, the basis for its selective expression in normal or cancer tissues remains incompletely understood. However, racial differences in methylation involved in BCa development, progression, or outcomes in African Americans may be implicated, are still unclear, and further research is needed.

Previously, the potential racial differences in DNA methylation in breast tumors were assessed by comparing differential methylation in African American and European American women according to ER and PR status and according to age (10). Among women diagnosed before age 50 and with tumors that were ER-negative/PR-negative, we found that African American women had a significantly higher frequency of methylation in four of five evaluated genes: *HIN-1* (79% in AA and 19% in EA), *TWIST* (67% and 16%), *CYCLIN D2* (64% and 19%), *RASSF1A* (76% and 29%) and *RAR-β*, (40% and 8%, ns); and were more likely compared to European American women to have three or more hypermethylated genes (80% *vs*. 0%, $p<0.005$). No differences in methylation patterns were evident between African American and European American women with ER-positive /PR-positive tumors or among older women (>50 years). A high frequency of *ESRα* methylation has also been reported in Indian and Chinese women (38, 39). Additionally, two other studies reported a higher frequency of methylation in young African American women when compared to Caucasian and European women (10, 40, 41). ESRα-negative phenotype and the mortality rate of BCa are higher in African American women (42), and ESRα-negative phenotype is correlated with promoter hypermethylation. The reasons for this disparity remain poorly understood but may be partially explained by differences in the epigenetic landscape. Thus, understanding the frequency of methylation in the promoter of *ESRα* and its effect on the clinicopathological characteristics and disease prognosis of BCa in women of other ethnic groups might aid in the diagnostics and treatment.

Therefore, this study examined whether there is a direct link between P0/P1 promoters of *ESRα* aberrant methylation and the risk of progression of fibrocystic and fibroadenoma benign tumors to BCa. We used pyrosequencing to measure the DNA methylation in the P0 and P1 promoters of *ESRα* in mammoplasty breast reduction (normal), fibrocystic and fibroadenoma (benign breast tumors), ER-positive and ER-negative BCa tissues, and human BCa cell lines. To our knowledge, this is the first study to investigate the association between aberrant methylation of the P0/P1 promoters of ESRα and the risk of progression from benign tumors to BCa.

Materials and Methods

Human breast tissues. With the Howard University Institutional Review Board approval (IRB-11-MED-75) and following HIPPA guidelines, we retrieved and coded a total of 130 formalin-fixed-paraffin-embedded (FFPE) breast tissues (28 normal mammoplasties, 31 fibrocystic, 34 fibroadenoma, 15 ER-positive and 22 ER-negative BCa) from the Howard University Hospital (HUH) Pathology Department archives, along with their associated clinicopathological data.

Immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining. Five-µm sections were cut from the FFPE blocks and mounted on Superfrost Plus microscope slides (Thermo Fisher Scientific, Waltham, MA, USA) for H&E

and IHC staining with specific monoclonal antibodies for nuclear ESRα (clone EP1, Dako, Carpinteria, CA, USA) as described previously (43). Areas containing at least 70% of cancer cells were marked by the pathologist and dissected for DNA and RNA extraction. DNA and RNA were extracted from the whole tissue sections of fibrocystic and fibroadenoma benign tissues.

Breast cancer cell lines. Two Caucasian-derived MCF-7 (ER-positive, PR-positive, HER2-negative) and MDA-MB-231 (ER-negative, PR-negative, HER2-negative), and one African American-derived HCC1806 (ER-negative, PR-negative, HER2-negative) BCa cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were cultured in RPMI-1640 medium without phenol red (Cellgro, Mediatech Inc., Manassas, VA, USA) supplemented with 10% charcoaled stripped fetal bovine serum and 1X antibiotics (both from HyClone Laboratories, Logan, UT, USA), and incubated in 5% CO_2/95% air at 37˚C.

DNA and RNA isolation. Genomic DNA and RNA were isolated from the tissues/dissected BCa cells using E.Z.N.A FFPE DNA kit (Omega Bio-TEK Inc., Norcross, GA, USA) and from BCa cell lines using the All-Prep DNA/RNA/ Protein Mini Kit (Qiagen Sciences Inc., Germantown, MD, USA) following the manufacturer's protocols. DNA and RNA concentrations were measured using a Nanodrop Spectrophotometer (Nanodrop, Wilmington, DE, USA) and stored at –80˚C.

Bisulfite modification. Following the manufacturer's protocol, two hundred-500 ng/μl of the isolated genomic DNA from FPPE tissues was modified using sodium bisulfite treatment EZ Methylation Kit (Zymo Research Inc., Irvine, CA, USA). Bisulfite modification was completed by DNA de-sulfonation in 0.3 mol/l NaOH at 37˚C for 15 min. Then, the DNA was precipitated and washed in 70% ethanol, dried, and resuspended in 50 μl of TE buffer (100 mM Tris-Cl, 1 mM EDTA, pH 8.3), and stored at –20˚C.

Polymerase chain reaction (PCR) and pyrosequencing primers. A bioinformatics approach was employed to identify the DNA sequences of P0 and P1 promoters of the human *ESRα* from the National Center for Biotechnology Information (NCBI) database. This process was accomplished using PCR primers designed and previously described in the work performed by Iwase *et al.* (42).

Qiagen PyroMark Assay Design Software version 2.0.2 was used for designing PCR and pyrosequencing primers to assess the methylation status of CpG in P0 promoter with an overall score of 92%. Primers for P1 were purchased from Qiagen (PM PyroMark CpG Assay) (Table I). The PCR primers were designed to assess the methylation status of CpGs within 2 kb from the transcription start site for *ESRα* and within 0.5 kb from the transcription start site for *RASSF1A* and *HIN-1*. The reverse primer was substituted with a 5' tailed unlabeled reverse primer and a biotinylated universal primer at a ratio of 1:9 in the PCR reaction to create a single-strand DNA template for the pyrosequencing reaction. Both one-step or two-step PCR reactions were performed using 2 μl of bisulfite-converted genomic DNA and different bisulfite PCR primer sets in a standard PCR reaction mix, as previously described (43). Amplification was conducted using Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA). For *HIN-1,* PCR reaction was performed in two steps, as previously described (44). The integrity of the PCR product was verified following electrophoresis in a 2% agarose gel with ethidium bromide staining at 140 volts for 30 min.

Quantitative analysis of DNA methylation using pyrosequencing. Biotinylated PCR products were immobilized onto Streptavidin Sepharose High-Performance beads (GE Healthcare Piscataway, NJ, USA), washed, denatured, and the biotinylated strands were released into an annealing buffer containing the sequencing primer. Bisulfite-converted methylated DNA and bisulfite-converted unmethylated DNA were used as positive and negative controls, respectively. Pyrosequencing was performed using the Pyro Mark Gold Q96 Reagents

Table I. *Polymerase chain reaction (PCR) and pyrosequencing primers.*

PyroMark PCR and sequencing primers for *ESRα* P0 and P1 promoters	
P0 Promoter primers	Forward: 5' GGGAAGTAGTTAGTAGGTAGGGTATTTG 3' Reverse: 5' Biotin-TCACTCCCCACTACCATTCAT 3' Sequencing: 5' AGGGTATTTGGTAGTTTTT 3' Sequence analyzed: 5'TTYGGTAGATAYGTAGTTGGGTTATTGTAT AGYGTTGGATGAATGGTAGTGGGGAGTG 3'
P1 Promoter primers	Qiagen Inc., Valencia, California Catalog Number: PM00024619
PyroMark PCR and sequencing primers for *RASSF1*	
	Forward: 5' GGGGGAGTTTGAGTTTATTGA 3' Reverse: 5' Biotin-CTACCCCTTAACTACCCCTTCC 3' Sequencing: 5' GGGTAGTATTAGGTTGGAG 3'
PyroMark PCR and sequencing primers for HIN1	
	Forward: 5' GGGGAGTTTATAGGAGTTGTAGGATAG 3' Reverse 1: AACCAAACCAACAAAACTTTCTCAA Reverse 2: 5' Biotin-U- ACCAAAACCCAATATAAAAAACCT3' Sequencing: 5' GGGTTAAGTAGAGTTTTAGGAG 3' Sequence Analyzed: 5'GGYGGYGAGTTTTATGGYGYGGGGGTTYGGGGYGYGYGGGGAATTTG 3'

(Qiagen) and the PyroMark Q 96 MD equipment (Qiagen). Each pyrosequencing reaction was repeated at least twice.

Gene expression analysis. Following the manufacturer's protocol, two-µg of isolated total RNA was converted to cDNA using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems). Reverse transcription was conducted in a Gene Amp PCR System 9700 thermo cycler (Applied Biosystems). The running condition was set as follows: 37˚C for 60 min, 95˚C for 5 min and stored at 4˚C.

Statistical analysis. Statistical analyses were performed using SPSS version 22 (IBM, SPSS, Chicago, IL, USA). Tests were two-tailed and $p<0.05$ was considered statistically significant. Data was plotted using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Student's *t*-test assuming unequal variance was used to compare the mean methylation level between promoters. A two-way ANOVA mixed model was used to assess methylation changes in normal and BCa tissues. Fisher exact test was used to determine the association between age and types of breast tissues. Correlation between methylation and gene expression was performed using Pearson's correlation. We identified cancer as methylation-positive if its methylation level exceeded a threshold defined as the average methylation in normal tissues +2SD. We used logistic regression analysis to compare ER-positive and ER-negative BCa with fibroadenoma [Response Variables (Fibroadenoma=0; ER-positive and ER-negative Breast Cancer=1)] adjusting for methylation in *ESRα, RASSF1A,* and *HIN1.* The association between methylation of P0 and P1 promoters and clinicopathological characteristics was calculated using Wald Chi-Square test. Linear regression was used to evaluate the association between the risk of fibroadenoma progression to ER-positive and/or ER-negative BCa, based on the methylation status of the investigated genes.

Results

Tissues characteristics. In this study, we assessed the methylation status of the *ESRα* promoter region in 130 human FFPE breast tissues (Table II). The mean age of the patients was 36±14.5, 49±10.9, 31±14.6, 60.8±13, and 45.3±11.2 years for mammoplasties, fibrocystic,

Table II. *Breast tissue types, sample numbers, and patients' ages at diagnosis.*

Types of breast tissues	Sample size (total 130)	Mean age/year (SD±)	Mean age/year range	≤50 years	>50 years
Mammoplasty reduction	28	36 (SD±14.5)	19-69	23	5
Fibrocystic	31	49 (SD±10.9)	29-83	18	13
Fibroadenoma	34	31 (SD±14.6)	13-76	31	3
ER-positive BCa	15	60.8 (SD±13)	39-82	4	11
ER-negative BCa	22	54.3 (SD±11.2)	31-74	9	13

fibroadenoma, and ER-positive, and ER-negative tissues, respectively. The mean age indicates that patients at younger age (≤50 years) are more likely to be diagnosed with fibrocystic changes and fibroadenoma than patients with BCa (p=0.003). However, there was no difference in the age of diagnosis between ER-positive and ER-negative BCa patients (p=0.491).

Methylation and expression of P0 and P1 promoters of ESRα in human breast tissues. Pyrosequencing measured the DNA methylation level in the P0 and the P1 promoters. The mean methylation levels in the P0 promoter were 29%, 25%, 23%, 31%, 28%, and in P1 promoter 30%, 29%, 33%, 28%, 25%, for mammoplasty, fibrocystic, fibroadenoma, ER-positive, and ER-negative breast tissues, respectively. For each type of breast tissue, the mean level of methylation in the P0 promoter was compared to that of P1 using an unpaired student's *t*-test. There was a significant difference between the methylation levels in the P0 and P1 promoters (p=0.0001), with higher DNA methylation in the P1 promoter in the fibroadenoma tissues (Table III, Figure 1).

There is a heterogeneity in the methylation levels within the promoters of *ESRα* in ER-positive and ER-negative cell lines. Methylation of *ESRα* P1 promoter in fibroadenoma may serve as a potential signature indicating the progression to BCa.

No significant difference was observed in the levels of DNA methylation in the P0 and P1 promotes of *ESRα* in mammoplasty (p=0.150), fibrocystic (p=0.073), ER-positive (p=0.660), and ER-negative (p=0.521) breast tissues. We further investigated the effect of P0 and P1 promoters' methylation on *ESRα* expression using RT-PCR (Figure 2). The expression of *ESRα* increased by 2-fold in fibrocystic, fibroadenoma, and ER-positive BCa tissues. Conversely, expression of *ESRα* was down-regulated in ER-negative BCa tissues. These results indicate that ESR*α* expression might be more influenced by the unmethylation at the distal promoter (P0) region than at the proximal promoter (P1) region. Thus, P0 promoter transcript is the primary *ESRα* transcript in fibroadenoma breast tissues. DNA methylation may be an additional molecular marker of the genetic heterogeneity in breast cancer and contributes to the more complex molecular characterization of benign and tumor tissues.

Methylation and activity of the P0 and P1 promoters of ESRα in breast cancer cell lines. Several prior studies using targeted methylation approaches for a few genes have suggested that racial variation exists in methylation between African Americans and non-African Americans (or Caucasians) (10, 43-46), and a whole-genome study using the Illumina 450K platform supports these studies (47). Therefore, DNA methylation in *ESRα* promoters was also evaluated using pyrosequencing in the Caucasian MCF7 (ER-positive, PR-positive, HER2-negative) and MDA-MB-231 (ER-negative, PR-negative, HER2-negative), and the African American HCC1806 (ER-negative, PR negative, HER2-negative) BCa derived cell lines. There were differences in the methylation levels within the promoters of *ESRα* in ER-positive and ER-negative cell lines and clinical cases. The main reason could be the patients' ethnicity, African American and Caucasian, from whom the cell lines were derived.

Table III. *Percentage (%) of DNA methylation levels in ESRα P0 and P1 promoters in different types of Caucasian- and African American (AA)-derived human breast cancer (BCa) cell lines and breast tissues.*

	Ethnicity	P0 promoter	P1 promoter	*p*-Value
BCa cell lines				
MCF7 (ER-positive, PR-positive, HER2-negative)	Caucasian	51.5%	15.5%	**0.001**
MDA-MB-231 (ER-negative, PR-negative, HER2-negative)	Caucasian	17.5%	7.0%	0.422
HCC1806 (ER-negative, PR-negative, HER2-negative)	AA	5.0%	6.0%	**0.036**
Breast tissue types				
Mammoplasty	AA	29%	30%	0.150
Fibrocystic	AA	25%	29%	0.073
Fibroadenoma	AA	23%	33%	**0.0001**
ER-positive	AA	31%	28%	0.660
ER-negative	AA	28%	25%	0.521

Statistically significant *p*-values are shown in bold.

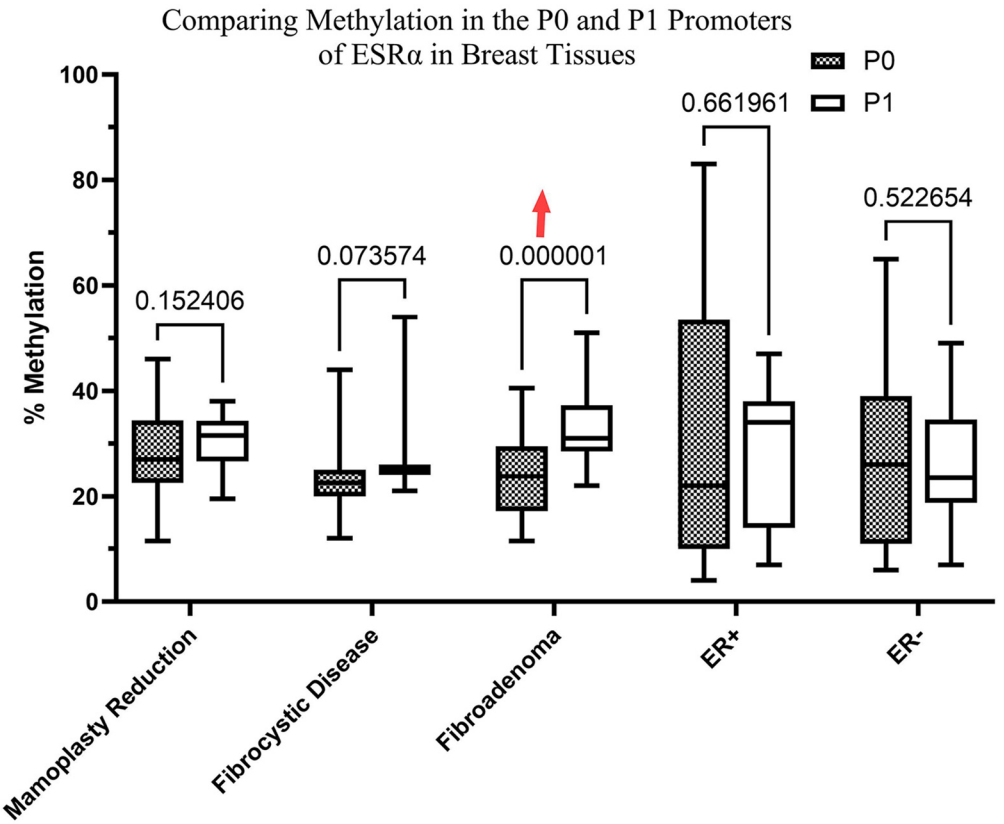

Figure 1. *Quantitative DNA methylation of ESRα promoters in different types of breast tissues. Y-axis represents the percentage of cytosine methylation assessed using pyrosequencing; X-axis represents the 5 breast tissue types. Box and whisker plot show the distribution of methylation in the P0 and P1 promoters in the 5 breast tissue types. Boundaries on each box represent values in the 25th and 75th percentile. Line within the box marks the median value. Whiskers (error bars) indicate the maximum and minimum value. Among the five breast tissue types, only fibroadenoma showed a significant difference in methylation in the P0 and P1 promoters with the P1 promoters showing high (red arrow) levels of methylation. ER-: ER-negative; ER+: ER-positive.*

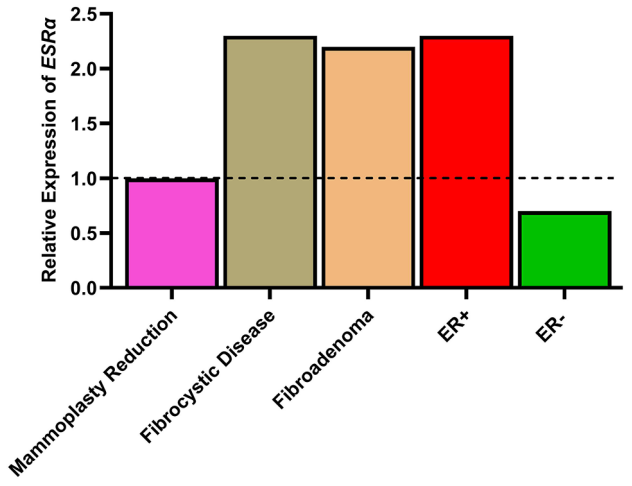

Figure 2. *Expression of ESRα in the different types of human breast tissues. RT-PCR expression of ESRα in breast tissues in relation to mammoplasty reduction (normal tissue). Enhanced ESRα expression in benign breast tissues might lead to elevated ESRα levels and increased cell proliferation, representing a significant early event in breast carcinogenesis. ER-: ER-negative; ER+: ER-positive.*

The mean methylation levels in the P0 promoter region were 51.5%, 17.5%, and 5%, and in the P1 promoter region were 15.5%, 7%, and 6%, in the MCF7, HCC1806, and MDA-MB-231 cell lines, respectively. For each cell line, an unpaired Student's *t*-test was used to compare the percentage of methylation between the P0 and the P1 promoters (Figure 3A). Both MCF7 (p=0.001) and HCC1806 (p=0.036) cell lines showed elevated levels of methylation in the P0 promoter when compared to the P0 promoter for each line. There was no significant difference in the methylation level between P0 and P1 promoters in the MD-MB-231 cell line (p=0.422). Furthermore, RT-PCR was performed to measure the expression of *ESRα* in the BCa cell lines. The analysis showed that the expression of *ESRα* was lower in the HCC1806 cell line relative to the MCF7 cell line, while the MDA-MB231 cell line showed no detectable *ESRα* expression (Figure 3B).

Correlation between ESRα promoter methylation and gene expression in human breast tissues. To assess the effect of *ESRα* promoter methylation in the five types of breast tissues, the percentage of methylation in the P0 and P1 promoter was correlated with the relative gene expression of *ESRα*. Pearson correlation revealed a negative correlation between methylation in the P0 promoter and gene expression in mammoplasty (r=–0.378; p=0.046) and ER-positive (r=–05425; p=0.036) tissues (Figure 4). No significant association was observed between *ESRα* methylation and gene expression in the P1 promoter in the above two breast tissue types; and in the P0 and P1 promoters in fibrocystic, fibroadenoma, and ER-negative breast tissues, which indicates that other mechanisms could be involved in the loss of *ESRα* expression in ER-negative BCa tissues.

To examine whether increased methylation of the P1 promoter of *ESRα* correlates with the loss of *ESRα* protein expression as observed in the fibroadenoma tissues, sections of 22 FFPE fibroadenoma tissues were assessed using IHC for *ESRα* expression. Positive nuclear staining for ERα was observed in all the stained samples, with differences in the percentage of reactive cells (1%-82%). The samples with 82% nuclear staining harbored an intraductal proliferative lesion (Figure 5). These differences in the intensity and distribution of the nuclear staining suggest that there is heterogeneity in the expression of the ERα in the fibroadenoma cells in each sample. Methylation may precede loss of protein expression for *ESRα*. In addition, these data suggested that many biological pathways and alterations are involved in ERα expression and regulation during BCa evolution.

Comparing methylation of P1 promoters of ESRα in fibroadenoma with ER-positive and ER-negative BCa. To examine aberrant DNA methylation patterns, the level of *ESRα* P0 and P1 promoters' methylation in fibroadenoma tissues were compared with that of ER-positive and ER-negative BCa. A significant difference in the level of methylation was observed in the P1 promoter in fibroadenoma tissues when compared with ER-negative BCa (p=0.001) with a higher level of methylation in the fibroadenoma tissues (Figure 6). No significant difference was observed between fibroadenoma and ER-positive BCa

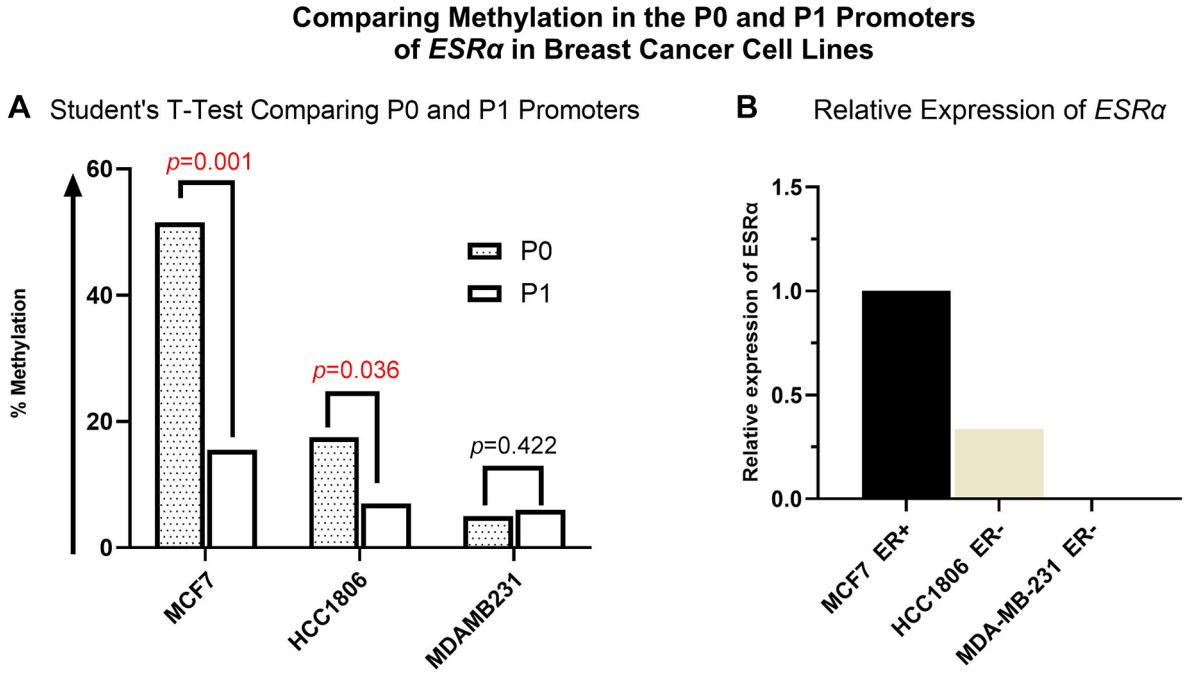

Figure 3. *Quantitation of DNA methylation of ESRα and its expression in BCa cell lines. (A) The bar graph shows the percentage of methylated cytosines in the P0 and P1 promoters of ESRα in BCa cell lines as obtained using pyrosequencing (Y-axis). The X-axis represents the ER-positive (MCF7) and ER-negative (HCC1806, MDA-MB231) BCa cell lines, and the result depicts the mean±standard deviation of two independent experiments. The p-value is indicated for ESRα gene (Mann–Whitney); (B) expression level of ESRα in breast cell lines was assessed using RT- PCR and normalized to MCF7. HCC1806 showed lower expression, while MDA-MB231 showed no expression of ESRα. ER-: ER-negative; ER+: ER-positive.*

(p=0.117). Furthermore, ER-positive and ER-negative BCa showed no difference in methylation in the P1 promoter (p=0.406). Ordinary one-way ANOVA also showed no significant difference in the level of methylation in the P0 promoter across the three-breast tissue types (p=0.256).

Methylation of RASSF1A and HIN1 in the benign fibroadenoma and BCa tissues. To define the genetic pathways that might clearly lead to fibroadenoma, we extended our analysis to include RASSFIA and HIN-1 genes. The *RASSFIA* gene encodes for a tumor suppressor and a regulator of cellular proliferation, apoptosis, and microtubule stability (48-50). Inhibition of *RASSFIA* expression through epigenetic mechanisms in the promoter have been considered as a biomarker for early detection of various types of cancers, including those originating from the breast (51, 52). An elevated level of *RASSF1A* methylation was reported in ER-positive DCIS and IDC tumors (53, 54). The *HIN-1* gene encodes for a secretory protein, which is a negative regulator of cell growth (55). Also, HIN-1 is a putative breast tumor suppressor gene that is highly expressed in normal human epithelial breast cells but downregulated in invasive and metastatic BCa (56, 57).

Therefore, after observing a pattern of DNA methylation in the P1 promoter of *ESRα* in fibroadenoma, ER-positive, and ER-negative tissues, the methylation status of both tumor suppressor genes *RASSF1A* and *HIN-1* was assessed in these three types of breast tissues. The mean methylation levels were 48% in fibroadenoma, 52% in ER-positive BCa, and 17% in ER-negative BCa tissues. The methylation level of *RASSF1A* (Figure 7) was higher in the fibroadenoma when compared with ER-negative BCa (p=0.0002). Elevated methylation level in *RASSF1A* was also observed in ER-positive when compared to ER-negative BCa (p=0.0004). No significant difference was observed between fibroadenoma and ER-positive BCa (p=0.487).

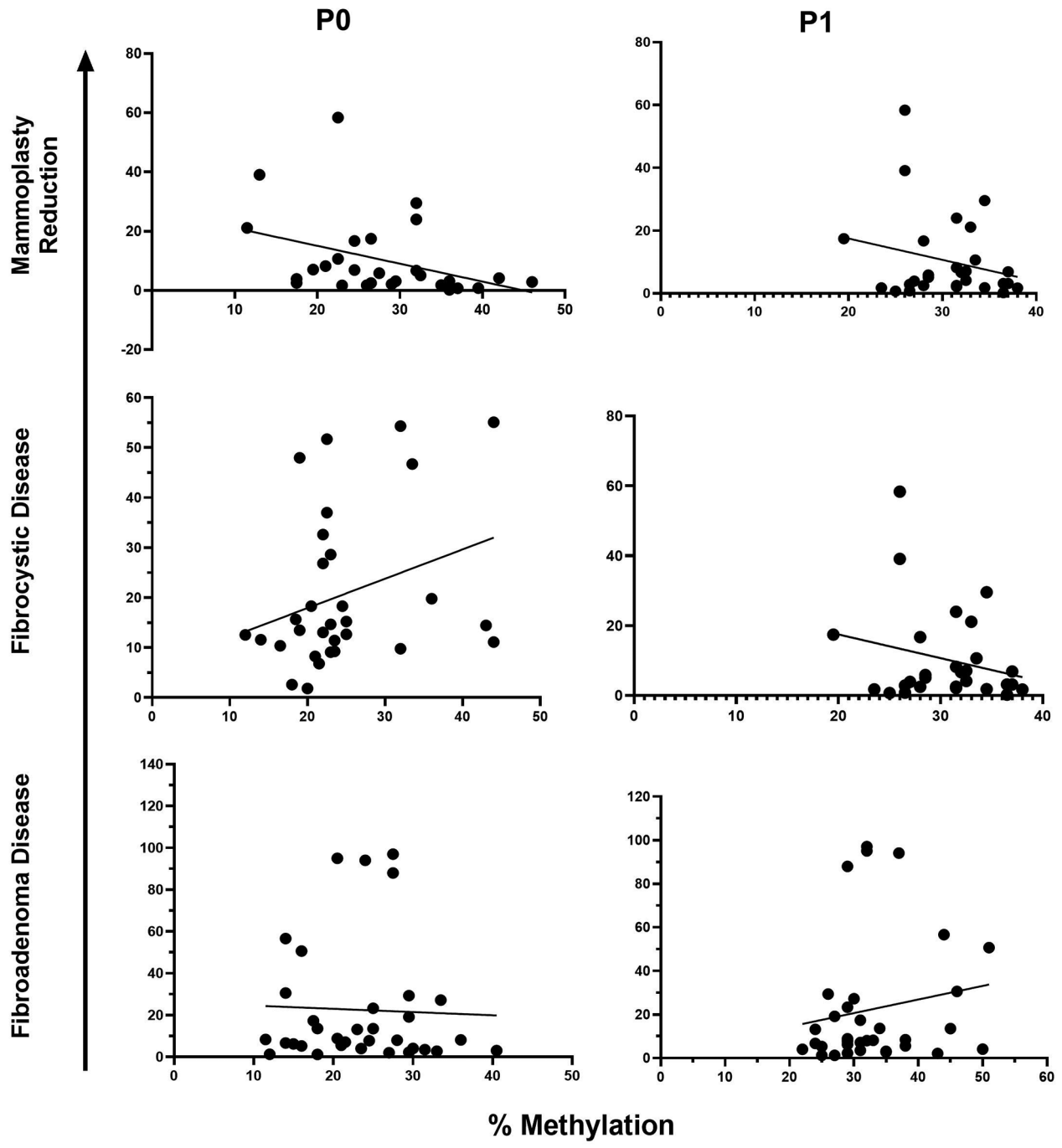

Figure 4. *Continued*

For *HIN-1*, the mean methylation level was 12% in fibroadenoma, 26% in ER-positive BCa, and 10% in ER-negative BCa. *HIN-1* was highly methylated in ER-positive BCa when compared to ER-negative BCa (p=0.046). In contrast, no significant difference was observed between fibroadenoma and ER-positive BCa (p=0.054) and

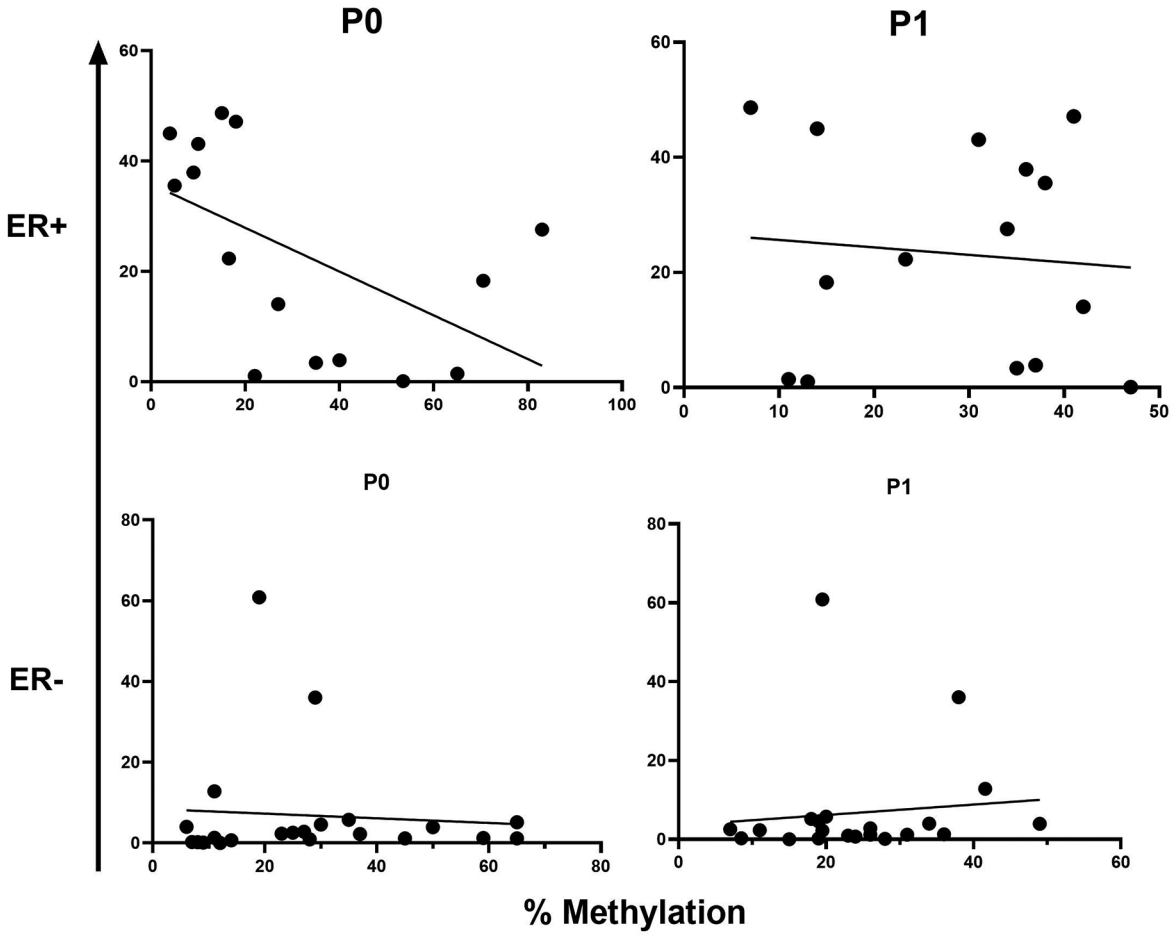

Figure 4. *Correlation between P0 and P1 ESRα promoter methylation and their expression in human breast tissues. Pearson correlation revealed a negative correlation between methylation level in the P0 promoter and gene expression in mammoplasty and ER-positive tissues.*

between fibroadenoma and ER-negative BCa (p=0.595) (Figure 8).

Logistic regression analysis of fibroadenoma to ER-positive and ER-negative BCa. Logistic regression analysis was performed to compare levels of DNA methylation in *ESRα*, *RASSF1A* and *HIN-1* genes between fibroadenoma and ER-positive and ER-negative BCa. The response variables for the logistic regression were (0) not cancer and (1) for cancer. Fibroadenoma was set as (0) and ER-positive as (1) when comparing fibroadenoma with ER-positive BCa, and fibroadenoma as (0) and ER-negative as (1) when comparing fibroadenoma with ER-negative BCa. A total of seven models with all combinations were assessed. Only samples from each breast tissue subtype for which methylation data from all three genes was available were included in the analysis. No significant difference was observed between the levels of DNA methylation in fibroadenoma and ER-positive BCa when the three genes were assessed as a single-panel model, two-panels model or a three-panels model. However, when all three genes were assessed in the three-panels model, *RASSF1A* was significant when adjusting or controlling for *ESRα* and *HIN-1* (Table IV). When comparing fibroadenoma with ER-negative BCa, the genes *ESRα* and *RASSF1A* showed significant difference in all tested models while *HIN-1* showed no significant

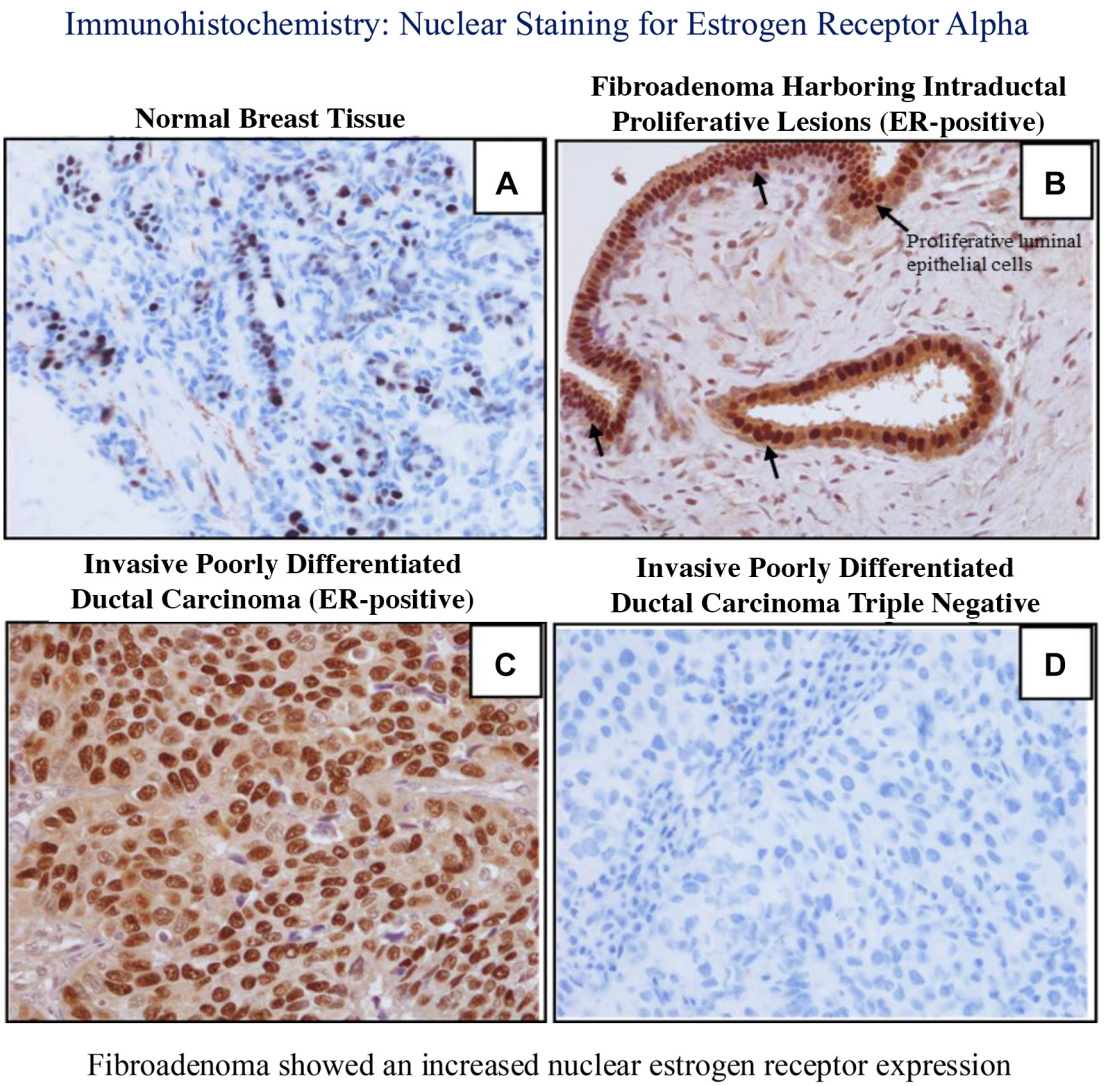

Figure 5. *Immunohistochemical nuclear staining (brown) of ESRα expression in (A) normal mammoplasty breast tissue; (B) fibroadenoma breast tissue with ER-positive proliferative luminal epithelial cells; (C) invasive ductal carcinoma with ER-positive; (D) invasive ductal carcinoma triple negative BCa.*

difference in all the tested models (Table V). These findings suggest that promoter methylation changed significantly in benign fibroadenoma tumors for two genes, *RASSF1A* when compared to both ER-positive and ER-negative BCa tumors, and *ESRα* only when compared to ER-negative BCa tumors. Methylation of the evaluated tumor-related genes can thus be considered as an early event in BCa progression.

To examine whether DNA methylation influences multiple gene networks rather than a single gene, further analysis was performed to assess if there was an interaction between DNA methylation in different genes (*ESRα, RASSF1A* and *HIN-1)* within the different types of breast tissues (Fibroadenoma, and ER-positive and ER-negative BCa). Two-way ANOVA univariate analysis of variance revealed a significant interaction between gene and breast tissue for two of the three types (p=0.001), including fibroadenoma and ER-positive BCa. ANOVA Mixed Model was further performed and revealed a

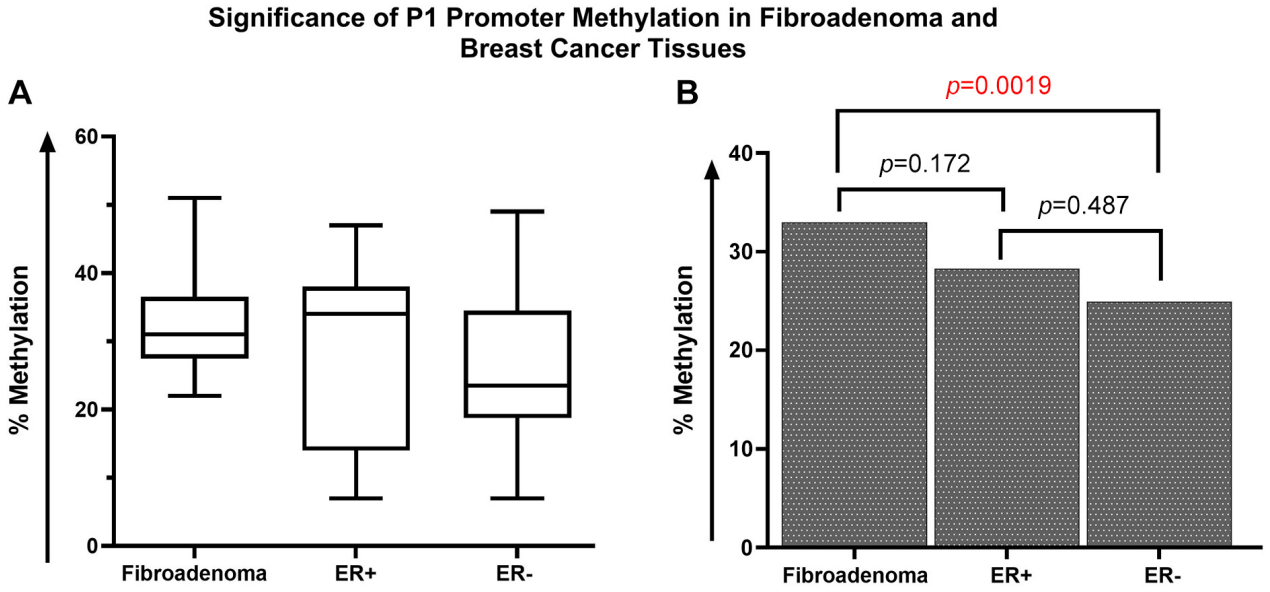

Figure 6. *Methylation of ESRα P1 promoter in fibroadenoma, ER-positive and ER-negative BCa tissues. (A) Quantitative DNA methylation analysis in three breast tissue types. Y-axis, percentage of methylated cytosines assessed using pyrosequencing; X-axis, breast tissue types. Box and whisker plot showing the distribution of methylation in the P0 and P1 promoters in the breast tissue types. Boundaries on the box represent values in the 25th and 75th percentile. Line within the box marks the median value. Whiskers (error bars) indicates the maximum minimum values; (B) Bar graph comparing the mean methylation levels. ER-: ER-negative; ER+: ER-positive.*

significant interaction between the *RASSF1A* gene with fibroadenoma and ER-positive BCa (p=0.004) (Figure 9).

Correlation of DNA methylation in the P0 and P1 promoters of ESRα with the clinicopathological characteristics. Statistical analysis showed an association between methylation levels of P0 and P1 promoters and the clinical characteristics of the ER-positive and ER-negative tumors (Table VI). DNA methylation in ER-positive BCa was associated with molecular subtypes (p=0.014) and grade (p=0.022). Tumors with unclassified molecular subtype (ER-positive, PR-negative, HER2-negative) had elevated levels of methylation in the P0 promoter compared with luminal B (ER-positive, PR-positive, HER2-positive) tumors (p=0.046). Tumors with grade 3 showed a borderline association with P1 promoter methylation compared to those with grade 2 (p=0.056). No association was observed with histology of the tumor, age, tumor size, stage, and lymph node status. For ER-negative BCa, there was a significant association between methylation and tumor size (p=0.018). Tumors ≤2 cm had elevated methylation levels in the P0 promoter compared to P1 promoter (p=0.001). No association was observed with the histology of the tumor, age, molecular subtypes, grade, stage, and lymph node status. No unknowns were included in the data analysis. Overall, these results showed that *ESRα* P0 promoter is methylated in the initial stages of breast carcinogenesis while the methylation in the P1 promoter occurs at late stages of BCa with poor prognosis.

Correlation of DNA methylation in promoters of RASSF1 and *HIN-1 with the clinicopathological characteristics*. There was an association between *RASSF1A* (p=0.0003) and *HIN1* (p=0.317) promoters' methylation with *ER* tumor status. No association was observed with histology of tumor, age, grade, stage, tumor size, or lymph node status (Table VII).

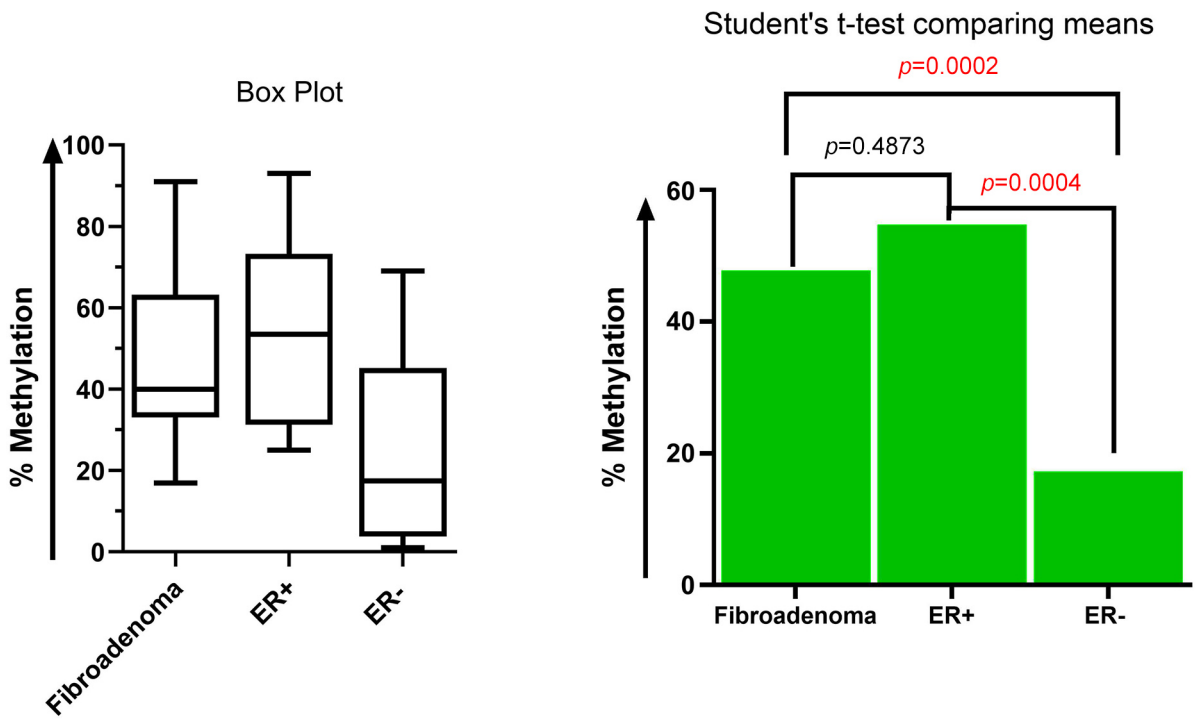

Figure 7. *Methylation of RASSFIA in fibroadenoma, ER-positive and ER-negative BCa tissues. (A) Quantitative DNA methylation analysis in breast tissues. Y-axis, percentage of methylated cytosines assessed using pyrosequencing; X-axis breast tissue types. Box and whisker plot showing the distribution of RASSF1A methylation in fibroadenoma, ER-positive and ER-negative BCa. Boundaries on the box represent values in the 25th and 75th percentile. Line within the box marks the median value. Whiskers (error bars) indicate the maximum and the minimum value; (B) Bar graph comparing the mean methylation levels. ER-: ER-negative; ER+: ER-positive.*

Discussion

Fibroadenoma is a benign breast disease that is commonly diagnosed in younger women (58) The clinical and histopathological diagnoses of benign breast tumors include developmental abnormalities, inflammatory lesions, epithelial and stromal proliferation as well as neoplasms (40, 59). *ESRα* promoter methylation is an epigenetic abnormality in cancer, and hypermethylation-mediated loss of *ER* expression could provide the cell with growth-promoting characteristics such as insensitivity to antigrowth signals (60).

In this study, we investigated whether there is a direct link between aberrant methylation in the P0 and P1 promoters of *ESRα* and the risk of progression of fibrocystic and fibroadenoma benign diseases to BCa. The percentage of DNA methylation levels in CpG islands of the P0 and P1 promoters of *ESRα* was quantified by using the high throughput pyrosequencing of DNA extracted from human ER-positive (MCF7) and ER-negative (HCC1806 and MDA-MB-231) BCa cell lines, and from different types of human breast tissues (mammoplasty breast reduction, benign breast diseases, and BCa). Quantitative RT-PCR was also performed to measure the expression of *ESRα, RASSF1A, and HIN1* in the BCa cell lines and breast tissues. The ER-positive (MCF7) and ER-negative (HCC1806) BCa cell lines had higher levels of DNA methylation in the P0 but not in the P1 promoter. However, the ER-negative MDA-MB-231 cell line showed low levels of methylation both in the P0 and P1 promoters. When the expression of *ESRα* was measured in these cell lines, relative to MCF7 cells, HCC106 cells showed downregulation of *ESRα* while MDA-MB-231 showed no expression of *ESRα*. Such data reveals that *ESRα* in MCF7 cells is transcribed from the P1 promoter. A study

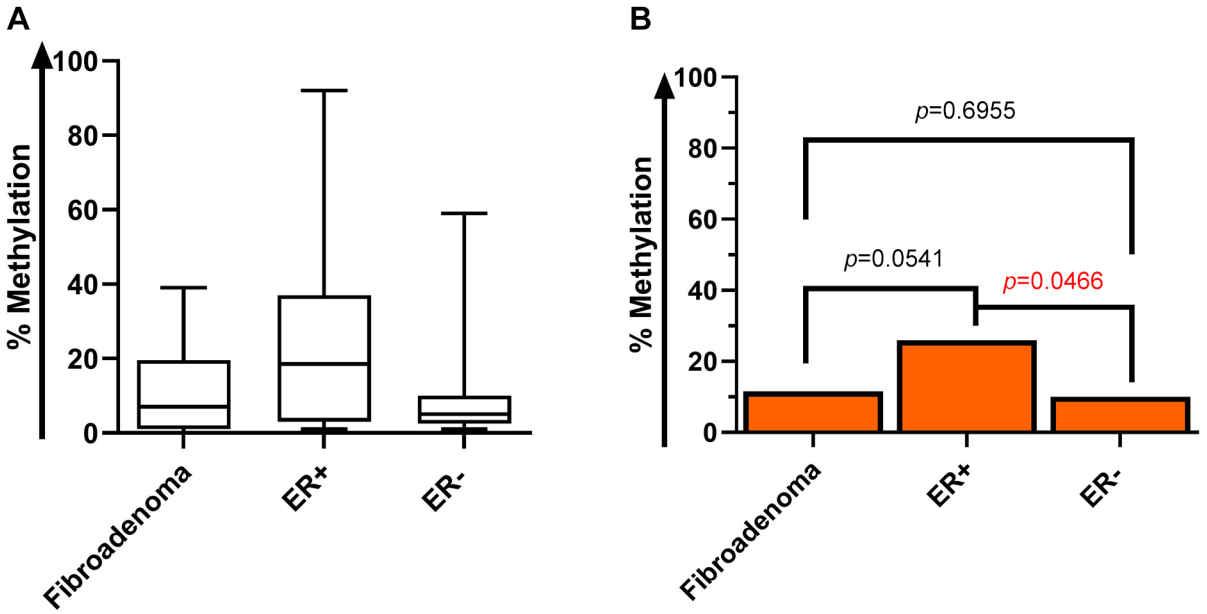

Figure 8. *Methylation of HIN-1 in fibroadenoma, ER-positive and ER-negative BCa tissues. (A) Quantitative DNA methylation analysis in breast tissues. Y-axis, percentage of methylated cytosines assessed using pyrosequencing; X-axis breast tissue types. Box and whisker plot showing the distribution of HIN1 methylation in fibroadenoma, ER-positive and ER-negative BCa. Boundaries on the box represent values in the 25th and 75th percentile. Line within the box marks the median value. Whiskers (error bars) indicate the maximum value and minimum value; (B) Bar graph comparing the mean methylation levels. ER-: ER-negative; ER+: ER-positive.*

by Tanimoto *et al.* (61) reported that in MCF7 cells in which ER is over-expressed, both the P0 and P1 promoters are used, and the transcript from the P0 promoter accounts for the enhanced expression. In contrast to Tanimoto *et al*. and Treilleux *et al.* (62, 63), findings suggest that the P1 transcript is the primary transcript in MCF7 cells. Similar to the results of this study, further evidence for partial DNA methylation in the P0 promoter in MCF7 cells has been reported (62, 64). A possible mechanism for methylation in the P0 promoter may be related to a pyrimidine-rich sequence in this region (62). Methylation of the 5th carbon position in the pyrimidine ring of a cytosine in a CpG island causes a change in chromatin structure abolishing the accessibility of transcription factors and promoting the formation of methyl-CpG binding domains. Methyl-CpG binding domains recruit additional silencing-associated proteins, which are all involved in gene silencing (62, 63). Another mechanism, reported by Yoshida *et al.* (64), involves the presence of a cis-element in the non-coding region, which functions as an enhancer sequence and interacts with the nuclear protein estrogen receptor promoter B associated factor-1 (ERBF-1). The binding of ERBF-1 onto this element is essential for basal-level expression of mRNA from the P0 promoter. It is the main determining factor for transcription and preferential usage of the P0 promoter. Deleting this element or inhibiting the protein complex that binds to this element leads to the complete suppression of P0 promoter activity. Other transcription factors that could be affected by methylation in the P0 promoter are Sp-1 and AP-1. The HCC1806 cell line showed a low methylation level in the P1 promoter, while the MDA-MB-231 cell line showed low methylation levels in both the P0 and P1 promoters. Both BCa cell lines are ER-negative, and the low level of methylation suggests that other mechanisms could be involved in the loss of estrogen receptors. For example, Koster *et al.* (65) reported that high expression of growth hormone-releasing hormone in HCC1806 cells leads to the activation of MAP-

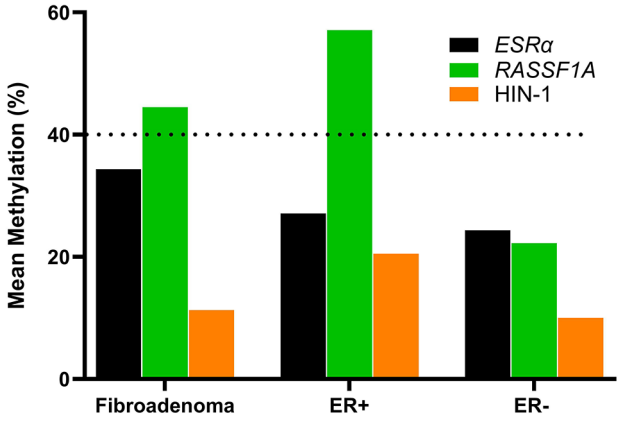

Figure 9. *Interaction between DNA methylation of different genes (ESRα, RASSF1A and HIN-1) within the different types of breast tissues. Bar graph showing mean methylation levels of ESRα, RASSF1A and HIN-1 in fibroadenoma, ER-positive and ER-negative BCa. ANOVA Mixed Model showed a significant interaction between fibroadenoma and ER-positive BCa with increased methylation in the promoter of RASSF1A (>40%) compared to ER-negative BCa (<40%). ER-: ER-negative; ER+: ER-positive.*

kinases ERK-1/2, and the hyperactivation of MAP-kinases is associated with loss of ER (66). Contrary to a previous study (67) in which methylation in the *ESRα* was associated with the loss of ER expression in the MBA-MB-231 cell line and demethylating treatment with 5-aza-2'-deoxycytidine induced re-expression ER, in this report, low levels of methylation were observed both in the P0 and P1 promoters, suggesting that methylation is not the only mechanism involved in the loss of ER in this cell line. Further evidence supporting our results comes from Hayashi's team, who did not observe ER re-expression after treating MDA-MDA-231 cells with increased concentrations of 5-aza-2'-deoxycytidine. The team concluded that the loss of the ER expression was not due to methylation in the gene but the loss of ERBF-1, again critical for suppression of the ER gene at the level of the P0 promoter. Another plausible mechanism is the loss of additional ERBF-1 protein family members that bind transcriptional regulatory elements in the P1 promoter, found to be essential for the transcription of the P1 promoter. At the same time, its binding site is crucial for the estrogen-independent activity of the P1 promoter (68). Furthermore, such inconsistency in these data from human BCa cell lines may be due to their dissimilar sources as well as changes in the nature of the cells during wide distribution or prolonged passaging.

Comparing methylation in the P0 and P1 promoters of ESRα in breast tissues. After evaluating the methylation status in the P0 and P1 promoters of ESRα for different human BCa cell lines (Figure 3), quantified using pyrosequencing, we repeated the same for breast tissues (Figure 1). Among the five types of breast tissues, only fibroadenoma showed a significantly higher level of methylation than the other tissues in the P1 promoter. The expression of *ESRα* was measured and relative to mammoplasty reduction, fibrocystic, fibroadenoma, and ER-positive BCa showed an approximately 2-fold increase in *ESRα* expression and low expression in ER-negative BCa (Figure 2). When *ESRα* expression was correlated with levels of methylation in the P0 and P1 promoters, a negative correlation was observed between methylation in the P0 promoter in the mammoplasty reduction tissue and ER-positive BCa (Figure 4). No correlation between methylation levels and *ESRα* expression was observed in the P1 promoter for any of the five types of breast tissues. Similar results of weak correlation between ESRα methylation and estrogen expression have also been published (69-71). Following this observation, the methylation in the P0 promoter was compared across the five types of breast tissues, and no significant difference was observed. Furthermore, several prior studies that used targeted methylation approaches for a few genes have suggested that racial variation exists in methylation between African Americans and non-African Americans (compared explicitly to Caucasians). We found differences in the methylation levels within the promoters of *ESRα* in ER-positive and ER-negative BCa cell lines derived from women of both populations, where patient ethnicity from whom the BCa cell lines were derived could be a major contributing factor to methylation differences. These racial differences could also help explain the differences in clinical biology and outcomes between the cell lines and breast tumor tissues. We are planning to replicate and expand our study, including more tumor

Table IV. *Logistic regression analysis comparing methylation levels in fibroadenoma with ER-positive breast cancer.*

Genes	*ESRα*	*RASSF1A*	*HIN-1*	*ESRα + HIN1*	*RASSF1A + HIN1*	*ESRα + RASSF1A*	*ESRα + RASSF1A + HIN1*
ESRα	0.098	----	----	0.164	----	0.066	0.129
RASSF1A	----	0.114	----	----	0.052	0.078	**0.044**
HIN-1	----		0.173	0.321	0.069	----	0.155

Statistically significant *p*-values are shown in bold.

Table V. *Logistic regression analysis comparing methylation levels in fibroadenoma with ER-negative breast cancer.*

Genes	*ESRα*	*RASSF1A*	*HIN-1*	*ESRα +HIN1*	*RASSF1A + HIN1*	*ESRα + RASSF1A*	*ESRα + RASSF1A + HIN1*
ESRα	**0.009**	----	----	**0.009**	----	**0.024**	**0.025**
RASSF1A	----	**0.022**	----	----	**0.005**	**0.015**	**0.016**
HIN-1	----	----	0.091	0.595	0.949	----	0.865

Statistically significant *p*-values are shown in bold.

samples from women and patients with BCa from additional different ethnic backgrounds (*i.e.*, with Hispanic and Asian descent) to compare with our current results.

The influence of menstrual cycle on ESRα transcription. In normal human endometrial tissue, it is well documented that there is an increase in the expression of ER during the proliferative phase of the menstrual cycle. Still, this expression decreases during the secretory phase (72). Hori *et al.* (73) reported similar findings with endometrial diseases. They concluded that the P0 promoter is frequently methylated during the secretory phase of the menstrual cycle. In contrast, the P1 promoter is methylated during the proliferative phase, and the transcript of the P1 promoter is predominant in precancerous endometrial tissues. Studies conducted on breast tissue b (74) reported an increase in ER expression during the luteal phase of the menstrual cycle. Those results revealed that the translation of ER could be influenced during the distinct phases of a normal menstrual cycle due to the concentration of estrogen in the blood. There is convincing evidence supporting this claim in pre-menopausal women and post-menopausal women. In pre-menopausal women expression of ER is associated with transcript from the P1 promoter. Whereas, in post-menopausal women, ER is associated with transcripts from the P0 promoter in which low estrogen levels in a milieu of tumor cells influences the transcription from the P0 promoter in ER-positive BCa (29). Quintas-Granados reported that methylation levels at *ESR*1 were associated with menopausal status, and in postmenopausal patients, the methylation levels were 1.5-fold higher than in premenopausal patients (75). Therefore, the negative correlation observed in this study between P0 methylation and the expression of ER in mammoplasty reduction (23 patients <50 years) and ER-positive BCa (11 patients ≥50 years) could be explained by the deferential usage of the P0 and P1 promoter during menstrual cycle and low estrogen concentration in the blood.

Significance of P1 promoter methylation in fibroadenoma and BCa. Fibroadenoma is a benign breast lump that is associated with younger age. Pathologically, fibroadenoma is a biphasic tumor that is composed of an epithelial and a stromal component (38). Even though fibroadenoma is considered a benign breast disease, in the past years, there has been growing evidence of fibroadenoma harboring carcinomas (76-80). Therefore, examining the methylation pattern of cancer-causing genes in fibroadenoma will elucidate possible epigenetic mechanisms involved in the development and progression

Table VI. *Association of average % methylation levels of ESRα P0 and P1 promoters with the clinicopathological characteristics of the ER-positive and ER-negative tumors.*

Clinicopathological characteristics	ER-positive Average % methylation=59%		ER-negative Average % methylation=53%	
	N	*p*-Value	N	*p*-Value
Histology of tumor		0.156		0.768
Adenocarcinoma	2			
Ductal carcinoma	2			
Infiltrating ductal carcinoma	11		19	
Medullary adenocarcinoma			1	
Medullary carcinoma			1	
Metaplastic carcinoma			1	
Age	15	0.072	22	0.149
Molecular subtypes		**0.014**		0.795
Luminal A	1			
Luminal B	6			
Triple Negative			15	
HER2 Type			4	
ER-positive, PR-negative, HER2-negative	5			
ER-positive, PR-positive, Unknown	2			
ER-positive, PR-negative, Unknown	1			
ER-negative, PR-positive, HER2-negative			3	
Tumor size		0.922		**0.018**
≤2 cm	5		8	
>2 cm	6		12	
Unknowns	4		2	
Grade		**0.022**		0.774
Grade I				
Grade II	5		1	
Grade III	8		20	
Unknown			1	
Stage		0.186		0.77
0	1			
1	2		6	
2	4		5	
3	4		7	
4	1		1	
Unknowns	3		3	
Lymph node		0.3		0.853
Lymph node positive	8		6	
Lymph node negative	2		12	
Unknowns	4		4	
Survival status				
Dead	3		12	
Alive	11		10	
Unknown	1			

N: Number of samples. Statistically significant *p*-values are shown in bold.

of BCa. In this study, an elevated methylation level was observed in the *ESRα* P1 promoter in fibroadenoma compared to the methylation levels of the P1 promoter in ER-positive and ER-negative BCa (Figure 6). The methylation patterns in fibroadenoma and ER-positive BCa showed a similar but weak association; however, compared with ER-negative BCa, methylation in fibroadenoma was significantly higher. To further

Table VII. *Relationship between RASSF1A and HIN-1 promoter methylation with clinicopathological characteristics.*

Clinicopathological characteristics	*RASSF1A*		*HIN-1*	
	N	*p*-Value	N	*p*-Value
Histology of tumor		0.713		0.203
Infiltrating ductal carcinoma	29		29	
Others	4		6	
Age		0.377		0.403
ER-status		**0.0003**		**0.032**
ER-positive	12		14	
ER-negative	22		21	
Tumor size		0.499		0.89
≤2 cm	12		15	
>2 cm	17		18	
Grade		0.599		0.157
Grade II	4		5	
Grade III	29		29	
Stage		0.995		0.287
1	7		8	
2	8		8	
3	11		10	
4	4		2	
Lymph node		0.307		0.927
Positive lymph node	13		13	
Negative lymph node	16		16	

N: Number of samples. Statistically significant *p*-values are shown in bold.

investigate fibroadenoma, ER-positive and ER-negative BCa, DNA methylation of two additional genes (*RASSF1A* and *HIN-1*) was assessed. *RASSF1A* regulates ER expression and function, and *HIN-1* is a putative tumor suppressor and cytokine and an apoptosis regulator by inhibiting cell growth. Methylation in *RASSF1A* in fibroadenoma was similar to that in ER-positive BCa but not in ER-negative BCa (Figure 7). In contrast, low methylation levels in *HIN-1* in fibroadenoma did not show a significant difference between ER-positive or ER-negative BCa (Figure 8). However, a difference was observed between the ER-positive and ER-negative BCa tissue levels of *HIN-1* methylation. Previously, we found significantly higher methylation in African Americans than in Caucasian women for four genes within a five-gene marker panel (HIN-1, TWIST1, CCND1, RASSF1A); these differences were only evident within ER-negative tumors and among women diagnosed before the age of 50 years (10).

Logistic regression analysis. Logistic regression analysis was performed to compare fibroadenoma with ER-positive and ER-negative BCa, adjusting for ESRα, RASSF1A, and *HIN-1* in seven logistic panel models. When fibroadenoma was compared with ER-positive BCa, methylation of *RASSF1A* had a greater influence on the progression of fibroadenoma to ER-positive BCa when all three genes were placed in a single logistic model, suggesting that the progression of fibroadenoma to ER-positive BCa requires methylation in all three genes. When fibroadenoma was compared to ER-positive BCa, *ESRα* and *RASSF1A* had a more significant influence on the progression of fibroadenoma to ER-negative BCa, while *HIN-1* did not, suggesting that the progression of fibroadenoma toward ER-negative BCa requires methylation in *ESRα* and *RASSF1A*. This result also revealed the epistatic *RASSF1A* regulation of estrogen receptor expression and function. Similar to the results of this study, other groups have also reported high methylation of *RASSF1A* in ER-positive BCa (44, 69, 81-84) and methylation of *ESRα*, *RASSF1A* and *HIN1* in fibroadenoma (85, 86). Another study suggested that *ER-α* promoter methylation is a biomarker for outcome prediction of cisplatin resistance triple-negative breast cancer (87).

Gao *et al.* (9) reported that *ESRβ* promoter methylation could be a potential indicator of malignant changes in breast cancer. Therefore, the future direction of our work is to perform a replicative study to assess methylation in the promoters of *ESRα* (P1), *ESRβ*, and *RASSF1A* on a larger sample size of fibroadenoma and to explore other mechanisms involved in the loss of ER expression in BCa.

Conclusion

Collectively, based on the results from the logistics regression analysis, our study partially supported the hypothesis which predicted that methylation of *ESRα* P1 promoter influences the progression of fibroadenoma to ER-negative BCa. However, methylation of *ESRα* promoters does not affect gene expression. Therefore, it is possible that methylation of *RASSF1A* could be an essential marker for breast cancer progression. Our future directions are to

perform a replicative study to assess methylation in *ESRα* P1 and *RASSF1A* promoters on a larger sample size in fibroadenoma and explore other regulatory mechanisms of loss of ER expression in breast cancer.

Conflicts of Interest

The Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' Contributions

Study conceptualization, Y.K., O.O.K., R.L.C., and R.L.D.; methodology, S.D., D.B., and T.J.N; validation, Y.K., B.K.A., S.D., and T.J.N.; statistical analyses, VA; formal analysis, Y.K., S.D., T.J.N., and B.K.A.; data curation, S.D., T.J.N.; writing – original draft preparation, Y.K., S.D., R.L.C and O.O.K; writing – review and editing, A.H.D., S.N., K.B., R.L.D., O.O.K., S.D., B.S., B.K.A. and D.A.S.; supervision, Y.K. All Authors have read and agreed to the published version of the manuscript. Y.K. and R.L.C. contributed equally to the conceptualization of the study.

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