Association of estrogen receptors’ promoter methylation and clinicopathological characteristics in Iranian patients with breast cancer

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Abstract

Background: Estrogens play a substantial role in the proliferation, progression and treatment of breast cancer by binding with two estrogen receptors, alpha and beta (ERα and ERβ). Resistance to endocrine therapy is a major problem in the treatment of breast cancers and, in some cases, may be related to loss of ER gene expression. We have already showed that ERα methylation occurs in high frequency and may be one of the important mechanisms for ERα gene silencing in a subset of Iranian primary sporadic breast cancers. In the other hand, the CpG Island methylation status of ERβ and the relationship between clinicopathological features and the pattern of ERβ methylation in sporadic breast cancer are still unknown, especially in Iranian women.

Methods: In this study, we examined the exact role of DNA methylation in the estrogen receptors, alpha and beta genes using Combined Bisulfite Restriction enzyme Analysis (COBRA) and Methylation specific polymerase chain reaction (MSP) methods in 34 tissue and 40 peripheral white blood cells in the breast cancers.

Results and Conclusions: ERα promoter methylation was identified in 29(72.5%) tissue samples and 35(87.5%) peripheral blood. Among these ERα-methylated cases, the co-occurring methylation of ER β promoter in peripheral blood and tissue samples was evident in 25 (71.4%) patient (P=0.56). Furthermore, ERβ promoter methylation was detected in 13(32.5%) tissue samples and 4(10.0%) peripheral blood specimens. Of these ERα-methylated cases, the co-occurrent methylation of ERβ promoter in the peripheral blood and tissue samples was evident in 1(7.7%) patient (P= 0.11). Based on COBRA analysis the percentage of DNA methylation at methylation-sensitive BstUI restriction site of the ERα promoter A ranged from 1% to 91%. The percentages at promoters A region showed a borderline associations with lymph node involvement (P=0.079, r=0.55) and a significant correlation with the grade of tumors (p= 0.27, r=0.65). No significant relation was found between ERα promoter and ERβ promoter methylation (Odds ratio =2.82, 95%, CI =0.28–28.5, P=0.36). The methylation of promoter ON was observed in only a subset of tumors without ER by IHC. In addition, we did not find any significant correlation between the prognostic factors such as grade, tumor size, lymph node involvement, and methylation status of this promoter. Our results indicate that methylation of ERβ promoter ON is not responsible for the loss of gene expression in of all breast tumors.

Key words: Estrogen receptor; CpG Island -; COBRA, Breast Tumors.

Introduction

Breast cancer is the most prevalent cancer in Iranian women who are about 10 years younger than their western counterparts (Harirchi et al., 2004; Mousavi et al., 2007). Determining Molecular subtypes of the breast cancer leads to better understanding of the
therapy purposes and the clinical behavior of these tumors (Pujol et al., 2004; Perou et al., 2000). Breast cancer is generally a hormone-dependent tumor. Estrogens can control the growth of breast cells by binding with estrogen receptors (ERs). Two major ERα and ERβ are expression in the normal breast epithelial tissue (Kuiper et al., 1996). These receptors act as ligand-inducible transcription factors and play a crucial role in the proliferation of cancerous cells and progression of breast tumors. Clinically, it is essential to assess ER for predicting the response to hormonal therapy and evaluation of patient prognosis (Merkel et al., 1989; Allred et al., 1998). Approximately 70% of breast cancer patients synthesize ERα protein and respond to therapy with antiestrogens such as tamoxifen (Thushangi et al., 2010; Hanstein et al., 2004). However, a fraction of receptor positive cells in breast cancer lose their receptors during tumor progression thus leading to an increase in poor clinical consequences (Yang et al., 2001).

The role of ER-β in the breast cancer remains unclear due to the relative scarcity of investigation. However, there is strong evidence that ERβ may act as a tumor-suppressor gene. A number of studies in humans demonstrate that ERβ expression is decreased in breast tumors. In the same line, Skliris et al (2003) indicated that a complete loss of ER-β expression was seen in 21% of invasive carcinomas (Thushangi et al., 2010; Bardin et al., 2004; Roger et al., 2001; Skliris et al., 2003).

In a remarkable proportion of breast cancers, absence of ER is a consequence of aberrant methylation of CpG islands (Yang et al., 2001; Lapidus et al., 1998). Specifically, studies of some breast cancer cell lines have shown that methylation of CpG islands is concerned with decreased expression of ERα protein (Ferguson et al., 1995; Ottaviano et al., 1994; Ushijima et al., 2005; Yoshida et al., 2000), and treatment of these cell lines with demethylating agents leads to restoration of expression (Lapidus et al., 1996). However, making these observations in human tumors has been confirmed to be more complicated. Although several studies have indicated significant relationship between the frequency of DNA promoter methylation and ERα status (Yan et al., 2001; Iwase et al., 1999; Fabianowska-Majewska et al., 2006), results generally suggested that methylation is not limited to tumors with the absence of ERα (Yoshida et al., 2000; Iwase et al., 1999) and null relationship between methylation and protein expression was found in some studies (Hori et al., 1999).

Some studies, on the other hand, indicated that ERβ promoter ON is highly methylated in breast cancer cell lines and DNA methylation is an important mechanism for ERβ gene silencing in the breast cancer (Zhao et al., 2003; Rody et al., 2005). In sum, knowledge of the exact mechanism of the absence of ER in the breast tumors could propose a great benefit for the treatment, prevention, and reduction of the mortality caused by cancers (Hori et al., 1999).

In this study, we examined the role of estrogen receptor genes DNA methylation in patients with breast cancer using quantitative and qualitative methods. We also evaluated the association of the pattern of DNA methylation status of ER promoters in breast tumor tissue with the pattern of DNA methylation in peripheral white blood cells.

**Material and methods**

**Peripheral blood and tissue Samples.**

A total of 34 tissues of breast tumor samples and 40 peripheral Blood specimens from women with sporadic breast cancer were obtained from the Day and Atieh Hospitals in Tehran, Iran. The study was conducted under research protocols approved by the University of Tarbiat Modares Institutional Review Board. Breast tumor tissues were obtained by surgical resection and transferred in liquid nitrogen to a -80°C refrigerator. Blood samples were also collected in 10 ml EDTA-containing tubes and stored in a -20°C refrigerator. Medical records and data were collected based on clinicopathological features, including age, tumor size, tumor stage, tumor grade,
histological type, hormone receptor status, nodal status, family and reproductive history.

**DNA isolation and bisulfite modification**

DNA was isolated from frozen tissue samples by the use of the Roche High pure PCR template preparation kit based on manufacturers protocol. In the meantime, DNA from blood samples was also isolated by salting-out method. Bisulfite conversion reaction was carried out as described previously (Khazamipour et al. 2009).

MTHFR promoter hypermethylation in testicular biopsies of patients with non-obstructive azoospermia: the role of epigenetics in male infertility.

Briefly 1 μg DNA was treated with 0.2 M NaOH for 10 min at 37°C. The DNA was then reacted with 10 mM hydroquinone and 3.5 M sodium bisulfate, PH 5.0. The conversion reaction was carried out under mineral oil at 58°C for 16 hours. Samples were then purified using Qiagen DNA purification columns. Recovered samples were incubated in 0.3 M NaOH for 15 min as alkaline desulfonation step at room temperature. After ethanol precipitation, DNA was dissolved in 40 ml water and used immediately for PCR amplification or stored in -20°C.

This bisulfite-treated DNA was then desalted with the Roche High pure PCR template preparation kit according to manufacturer’s recommendations and eluted into approximately 30 μl sterile water. The DNA was subsequently precipitated by 10 M ammonium acetate with ethanol after desulfonation and resuspended in sterile water. The Control DNA sample was methylated using Sss1 methyltransferase (New England BioLabs) according to the manufacturer’s protocol and used as methylated, positive control for MSP reactions.

**Analysis of ER promoter methylation with Methylation specific polymerase chain reaction (MSP)**

ERα gene promoters A-F are distinguished so far, the transcript from promoter region A was utilized in both normal and cancerous breast tissue (Koš et al., 2001; Hayashi et al., 1997). First PCR amplification using methylation and unmethylation specific primers was performed in order to analyze the CpG Island methylation status of this promoter in 34 cancerous mammary tissue and 40 peripheral blood samples. PCR reaction for the methylated primer set (ERαAMF and ERαAMF) Carried out in a total volume of 25μl containing 7.5 μl DDW, 12.5 Ampliqon master mix. 3 μl (100 ng) DNA and, 1mM of each primer. PCR reaction underwent initial denaturation at 95°C for 5 min, and 35 cycles of the following profile: 30 s at 94°C, 40 s at 58°C, and 30 s at 72°C, and a final extension step at 72°C for 7 min. also, for unmethylated primers (ERαAUF and ERαAUR), an initial denaturing step at 95°C for 5 min was followed by 32 cycles at 94°C for 50s, 48°C for 30s, 72°C for 20s, and a final extension step at 72°C for 3 min. The reaction was performed in a total volume of 25μl containing 9.8 μl DDW, 12.5 Ampliqon master mix, 1.5 μl (100 ng) DNA, 0.5Mm of each primer and 4% DMSO.

Additionally, Promoter ON is considered as the most important promoter of ERβ gene and is related to several types of cancer including breast cancer (Xue et al., 2007). Second PCR amplification was carried out to analyse the CpG Island methylation status of ON promoter by the use of methylation and unmethylation specific primers. PCR for the methylated primer set (ERβMF and ERβMF) was performed in 25 μl of reaction mixture containing 12 μl DDW, 2.5 mM MgCl2, 4 mMdNTP, 2.5 μl PCR buffer (10x), 1.5 μl (100ng) DNA, 1 mM of each primer, 0.5 μl Taq polymerase (Cinnagen, Iran). An initial denaturing step at 95°C for 5 min was followed by 40 cycles at 94°C for 30s, 61°C for 45s, 72°C for 30s, and a final extension step at 72°C for 4 min. Also, for the unmethylated primers set (ERβUF and ERβUR) an initial denaturing step at 95°C for 5 min was followed by 35 cycles at 94°C for 30s, 54.7°C for 30s, 72°C for 20s, and a
final extension step at 72°C for 5 min. The reaction was carried out in a total volume of 25μl containing 15.8 μl DDW, 1.5 mM MgCl2, 2 mM dNTP, 2.5 μl PCR buffer (10x), 1 μl (100ng) DNA, 1 mM of each primer, 0.2 μl Taq DNA polymerase (Cinnagen Iran).

All the primers used for the analysis methylation status were designed using MethPrimer (Li et al., 2002). Primers used for PCR reactions are summarized in table 1. The PCR products were electrophoresized on a 2% agarose gel and stained with ethidium bromide and DNA treated with SssI bacterial methylase was used as a positive control.

**Analysis of ER promoter methylation by Combined Bisulfite Restriction enzyme Analysis (COBRA) assay**

To determine a sensitive quantification of DNA methylation levels, COBRA was set up for 12 samples demonstrated both methylated and unmethylated alleles in ERα promoter A.

The PCR reaction prior to enzymatic digestion carried out in a total volume of 25μl containing 9.8 μl DDW, 10 μl Qiagen Master Mix, 5μl (250ng) DNA, 0.5 mM of each primer, 2 μl Qiagen dye. An initial denaturing step at 95°C for 3 min was followed by 35 cycles at 92°C for 30s, 50°C for 40s, and a final extension step at 72°C for 10 min. Then 10μl of bisulfite-converted PCR product were subjected to enzymatic digestion, using 1μl (10U) of the enzyme BstUI, in a reaction containing 2 μl enzyme buffer, and 7μl of double distilled water. The reaction was incubated at 37°C over night and followed by incubation in 65°C for 20 minutes to deactivate the enzyme. BstUI normally digests CGCG site when methylated and can thus differentiate the two alleles. In this reaction, BstUI recognizes a CGCG sequence in the PCR product of 208bp and produces two fragments with 50 and 158bp long.

Control DNA sample was methylated using SssI methyltransferase (New England BioLabs) according to the manufacture’s protocol and used as methylated positive control. The products of enzymatic digestion were separated by electrophoresis on a 2% agarose gel and, stained by ethidium bromide. Densities of digested and undigested PCR products in COBRA were measured after normalization to calculate the percentage of methylated and unmethylated alleles.

**Table 1 Primers used for MSP and COBRA s**

<table>
<thead>
<tr>
<th>Promoter</th>
<th>speciation</th>
<th>primer name</th>
<th>primer sequence</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>Methylation Specific</td>
<td>ERαAMF</td>
<td>5'-GATACGTTTTGATTTTGCTGC-3'</td>
<td>58°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ERαAMR</td>
<td>5'-CGAACGATTTCAAAACTCCAACT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unmethylation</td>
<td>ERαAUF</td>
<td>5'-GGATATGTTTTGAATTTTTGTT-3'</td>
<td>58.7°C</td>
</tr>
<tr>
<td></td>
<td>Specific</td>
<td>ERαAUR</td>
<td>5'-ACAAACAATTTCAAAACTCCAACT-3'</td>
<td></td>
</tr>
<tr>
<td>COBRA assay</td>
<td></td>
<td>ERαCoF</td>
<td>5'-GTTTTGGATTTTTTTGTTTG-3'</td>
<td>50°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ERαCoR</td>
<td>5'-AACTTACTACTATCCAAATACACCTC-3'</td>
<td></td>
</tr>
<tr>
<td>ERβ</td>
<td>Methylation Specific</td>
<td>ERβMF</td>
<td>5'-GGGGGATTATTTCTGTTGC-3'</td>
<td>61°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ERβMR</td>
<td>5'-AAATACGAAACGTGTCTTTCC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unmethylation</td>
<td>ERβUF</td>
<td>5'-GGGGGATTATTTCTGTTTG-3'</td>
<td>54.7°C</td>
</tr>
<tr>
<td></td>
<td>Specific</td>
<td>ERβUR</td>
<td>5'-AAATACAAACACATCTTTTCC-3'</td>
<td></td>
</tr>
</tbody>
</table>
Statistical analysis
Chi-squared analysis was performed in order to determine the impact of ER methylation status of CpG islands on prognostic factor, such as malignancy grade, tumor size, lymph node involvement, hormone receptors, HER-2 status, and nuclear accumulation of P53 in patients. In addition, Pearson Correlation was calculated to find a relationship between the percentage of ER methylation status of CpG Islands and clinicopathological features of tumors.

Results
Demographic and clinicopathological findings
The association between ER methylation and clinicopathological features of the 34 breast tissue tumors are demonstrated in table 2. DNA methylation was evident for 87.5% of breast tumors at ERα promoter A and 38.23% at ERβ promoter ON with MSP assay.
Analysis of CpG Island methylation status of ERα and ERβ in the primary tumors demonstrated that DNA methylation at promoter A and promoter ON were not significantly related to IHC markers including estrogen receptor, progesterone receptors, TP53 and HER2. Furthermore, we didn’t find any significant correlation between factors such as, grade, tumor size, lymph node involvement and methylation status at both promoters (Table 2).

<table>
<thead>
<tr>
<th>Table 2 Demographic and clinicopathological findings</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Feature</th>
<th>ERα Methylated</th>
<th>Unmethylated</th>
<th>p-Value</th>
<th>ERβ Methylated</th>
<th>Unmethylated</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size (cm) n= 29</td>
<td>n= 5</td>
<td>0.19</td>
<td>n=13</td>
<td>n= 21</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>MEAN ± SD</td>
<td>2.71±1.2</td>
<td>3.7±2.6</td>
<td>2.50±1.37</td>
<td>3.07±1.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodes involved n=28</td>
<td>n=5</td>
<td>0.75</td>
<td>n=12</td>
<td>n= 21</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>MEAN ± SD</td>
<td>3.68±4.6</td>
<td>3.0±3.0</td>
<td>3.67±3.9</td>
<td>3.52±4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor grade</td>
<td>n= 29</td>
<td>n=5</td>
<td>0.43</td>
<td>n=13</td>
<td>n= 21</td>
<td>0.85</td>
</tr>
<tr>
<td>2</td>
<td>17(58.6)</td>
<td>2(40)</td>
<td>7(53.8)</td>
<td>12(57.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>12(41.4)</td>
<td>3(60)</td>
<td>6(46.2)</td>
<td>9(42.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor type</td>
<td>n= 29</td>
<td>n=4</td>
<td>0.55</td>
<td>n=13</td>
<td>n= 20</td>
<td>0.97</td>
</tr>
<tr>
<td>Ductal</td>
<td>25(86.2)</td>
<td>3(75.0)</td>
<td>12(84.6)</td>
<td>17(85.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lobularl</td>
<td>4(13.8)</td>
<td>1(25.0)</td>
<td>2(15.4)</td>
<td>3(15.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER status</td>
<td>n= 28</td>
<td>n=5</td>
<td>0.94</td>
<td>n=13</td>
<td>n= 20</td>
<td>0.50</td>
</tr>
<tr>
<td>positive</td>
<td>22(78.6)</td>
<td>4(80)</td>
<td>11(84.6)</td>
<td>15(75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>6(21.4)</td>
<td>1(20)</td>
<td>2(15.4)</td>
<td>5(25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR status</td>
<td>n= 29</td>
<td>n=5</td>
<td>0.88</td>
<td>n=13</td>
<td>n= 21</td>
<td>0.23</td>
</tr>
<tr>
<td>positive</td>
<td>24(82.8)</td>
<td>4(80)</td>
<td>12(92.30)</td>
<td>16(76.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>5(17.2)</td>
<td>1(20)</td>
<td>1(7.7)</td>
<td>5(23.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2</td>
<td>n= 24</td>
<td>n=4</td>
<td>0.11</td>
<td>n=12</td>
<td>n=16</td>
<td>0.57</td>
</tr>
<tr>
<td>Positive</td>
<td>8(33.3)</td>
<td>3(75.0)</td>
<td>4(33.3)</td>
<td>7(43.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>16(66.7)</td>
<td>1(25)</td>
<td>8(66.7)</td>
<td>9(56.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53</td>
<td>n=14</td>
<td>n=2</td>
<td>0.24</td>
<td>n=6</td>
<td>n=10</td>
<td>0.42</td>
</tr>
<tr>
<td>Positive</td>
<td>8(57.1)</td>
<td>2(100)</td>
<td>3(50)</td>
<td>7(70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>6(42.9)</td>
<td>0(0)</td>
<td>3(50)</td>
<td>3(30)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Methylation of ERs

Methylation of ERα promoter A was observed in 35/40 (87.5%) of peripheral blood specimens whereas 13/40 (32.5%) of ERα promoter A was unmethylated in these samples. In the corresponding 34 tumor tissue samples, 29/34 (85.3%) of ERα promoter A was methylated, and 17/34 (50%) of them were unmethylated. Furthermore, Methylation of ERβ promoter ON was not observed in the peripheral blood specimens, whereas 4/40 (10%) of ERβ promoter ON are methylated in these samples. In tumor tissue samples, 13/34 (38.2%) of ERβ promoter ON was methylated. However in 100% (34/34) of samples the unmethylated allele was detected.

To determine if there is an association between ERα and ERβ promoter methylation patterns, we analysed the methylation status of the both promoter in the 34 tumor breast tissues. ERβ promoter methylation was identified in 13 tumors (38.2%). Among these 13 ERβ-methylated cases, 12 cases had also ER promoter-methylation. Also, it was founded that DNA methylation level at ERα promoter A was 2.30 folds higher than that of promoter ON in breast tissue tumors. The relationship between ERα methylation and ERβ methylation is shown in Table 2.

Additionally, in order to examine the relationship between ER promoter methylation patterns in peripheral blood and tissue samples we analysed the methylation status of the both promoter in 40 peripheral blood and 34 tissue samples. ERα promoter methylation was identified in 29(72.5%) tissue samples and 35(87.5%) peripheral blood. Among these ERα-methylated cases, the co-occurrence of ER promoter methylation in peripheral blood and tissue samples was evident in 25 (71.4%) patient. Furthermore, ERβ promoter methylation was detected in 13(32.5%) tissue samples and 4(10.0%) peripheral blood specimens. Of these ERα-methylated cases, the Co-occurrence methylation of ERβ promoter in peripheral blood and tissue samples was evident in 1(7.7%) patient. The relationship between ER promoter methylation in peripheral blood and tissue samples are demonstrated in Table 4 and 5.

<table>
<thead>
<tr>
<th>Promoters ERβ</th>
<th>ERα Unmethylated (%)</th>
<th>ERα Methylated (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmethylated</td>
<td>4(80)</td>
<td>17(58.6)</td>
<td>21(61.8)</td>
</tr>
<tr>
<td>Methylated</td>
<td>1(20)</td>
<td>12(41.4)</td>
<td>13(38.2)</td>
</tr>
<tr>
<td>Total</td>
<td>5(14.7)</td>
<td>29(85.3)</td>
<td>34(100.0)</td>
</tr>
</tbody>
</table>

Odds ratio = 2.82, 95% confidence interval = 0.28–28.5. Pearson Chi-Square test; P = 0.36

<table>
<thead>
<tr>
<th>Promoters ERα/Blood</th>
<th>No tissue (%)</th>
<th>ERα Unmethylated /Tissue (%)</th>
<th>ERα Methylated/ Tissue (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmethylated</td>
<td>0(0)</td>
<td>1(20)</td>
<td>4(13.8)</td>
<td>5(12.5)</td>
</tr>
<tr>
<td>Methylated</td>
<td>6(100)</td>
<td>4(80)</td>
<td>25(71.4)</td>
<td>35(87.5)</td>
</tr>
<tr>
<td>Total</td>
<td>6(15.0)</td>
<td>5(12.5)</td>
<td>29(72.5)</td>
<td>40(100)</td>
</tr>
</tbody>
</table>
Table 5 Relationship between ERβ Methylation at promoter ON in peripheral blood and tissue samples. Pearson Chi-Square test; P = 0.11

<table>
<thead>
<tr>
<th>Promoters ERβ /Blood</th>
<th>No tissue (%)</th>
<th>ERβ Unmethylated /Tissue (%)</th>
<th>ERβ Methylated/ Tissue (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmethylated</td>
<td>4(66.7)</td>
<td>20(95.2)</td>
<td>12(92.3)</td>
<td>36(90.0)</td>
</tr>
<tr>
<td>Methylated</td>
<td>2(33.3)</td>
<td>1(4.8)</td>
<td>1(7.7)</td>
<td>4(10.0)</td>
</tr>
<tr>
<td>Total</td>
<td>6(15.0)</td>
<td>21(52.5)</td>
<td>13(32.5)</td>
<td>40(100)</td>
</tr>
</tbody>
</table>

Figure 1 MSP analysis of ERα promoter A (A) and ERβ promoter ON(B) using Unmethylated and methylated sequence-specific primers in the peripheral blood samples. The products were separated by polyacrylamide gel electrophoresis. A, from right: positive control (CL/pos; Sss1 treated DNA), 100bp marker, negative control (CL/neg), blank, and 6 blood samples where 4 of them showed a methylated allele (120bp). B, from right: 100bp marker, blank, positive control, negative control (Sss1 treated), 3 blood samples, all showing the unmethylated allele (157bp).

Combined Bisulfite Restriction enzyme Analysis (COBRA) assay

In order to quantitatively analyse the methylation pattern of the ERα promoter A in 12 tissue samples where both methylated and unmethylated bands were present in the image of the polyacrylamide gel electrophoresis on MSP test, we performed COBRA. In COBRA, the fragment amplified from methylated DNA can be identified as digestible bands with restriction enzyme BstUI, because of the retention of methyl-cytosine residue at C nucleotides of CpGs even after bisulfite-treatment. Among these sample 11/12 (91.66 %) revealed methylation in the region analyzed by COBRA (Fig. 2).

The ratio between the BstUI-cleaved PCR product and the total amount of PCR product was employed to determine the percentage of fully methylated BstUI sites in a genomic DNA (ZhXiong, 1997). To examine the relationship between the percentage of methylation in ERα promoter A and clinicopathological factors such as grade, tumor size, lymph node involvement, a correlation analysis was performed. The results as appeared in Table 3 show that the correlation between these factors is intermediate (0.3<r<0.7). This indicates that any of these factors can variously correlate to the percentage of methylation status in ERα promoter A. The percentage of DNA methylation at promoter A region was significantly correlated to grade tumors (p=0.27, r=0.65; Table 6, Fig.2 B). Additionally, the percent of DNA methylation at promoters A region showed borderline associations with lymph node involvement (p=0.079, r=0.55; Table 6, Fig. 2 D).
Figure 2 COBRA for analyzing the ERα promoter A methylation status. A, PCR-amplified A promoter fragment from a representative patient. The digested products were separated by polyacrylamide gel electrophoresis and enzymatic digestion performed by BstUl on the 208bp PCR product. From left: 100bp size marker, 3 tissue samples showing both the digested and undigested bands (methylated and unmethylated allele respectively), Sss1 treated positive control (completely digested, 158bp). B, Sequence of A CpG island amplified COBRA. BstUl normally digests CGCG site when methylated and can thus differentiate the methylated and unmethylated. C, Association between the percentage of methylation and size of the tumors. D, Association between the percentage of methylation and grade of the tumors.

Table 6 Correlation between percentage of methylated BstUl sites at promoter A and clinicopathological finding

<table>
<thead>
<tr>
<th>Percentage of Methylation</th>
<th>Lymph node involvement</th>
<th>Grade tumors</th>
<th>Size of the tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation</td>
<td>0.55</td>
<td>0.65</td>
<td>0.52</td>
</tr>
<tr>
<td>P-Value</td>
<td>0.07</td>
<td>0.02</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Discussion

Resistance to endocrine therapy is a major problem in the treatment of breast tumors and, in some cases, may be associated with silencing of ER gene expression. Methylation within 5′ CpG islands of target genes is one of the potential mechanisms for silencing of gene expression. Such aberrant DNA methylation
has been related to lack of expression of several tumor suppressor genes (Graff et al., 1995). In an attempt to better understand the role of epigenetic events in the breast cancer development and progression, we have examined the methylation status of ERα promoter A and ERβ promoter ON and their association with pathological findings.

In this study, we demonstrated that methylation in ERα promoter A occurred in 85.3% of the breast cancer cases and is not correlated with IHC markers including estrogen receptor, progesterone receptors, TP53 and HER2. Furthermore, we did not find any significant correlation between prognostic factors such as grade, tumor size, lymph node involvement, and methylation status of promoter A. In a study conducted by Parrella et al. (2004), methylation of ERα promoter A was observed in 46% of the samples by the use of this study’s set of primers and no significant correlation between the methylation status was found with regard to prognostic factors and the hormone receptors (Parrella et al., 2004).

Aberrant methylation of CpG islands promoter is demonstrated as a frequent mechanism for silencing the numerous gene expression involved in several functions, including cell-cycle regulation (p16INK4a and cyclin D2), cell adhesion (E-cadherin), regulation of cell transcription (HOX5A), DNA repair (BRCA1 and GSTP1), receptor-mediated cell signaling (RAR-β and THR-β), regulation of cell transcription (HOX5A) in breast tumors (Mehrotra et al., 2004). However, the complexity of ERα promoter is greater than that of many other genes (Kos et al., 2001) and various ERα DNA methylation patterns in breast tumors have been reported in Turkish (Buyru et al., 2009), Chinese (Zhao et al., 2008), North American and Korean (Lee et al., 2008), Indian (Mirza et al., 2007), and American (Wei et al., 2008) women with breast tumors.

Like ERα, ERβ has a complicated 5’ region, with two distinct promoters, named OK and ON. The function of ERβ in breast tissue was not completely determined. However more recent observations suggest the substantial role of ERβ as a tumor-suppressor gene (Rody et al., 2005). In this study, we demonstrated that methylation in ERβ promoter ON occurred in 38.2% of the breast cancer cases and was not correlated with IHC markers, including estrogen receptor, progesterone receptors, TP53 and HER2. Furthermore, we did not find any significant correlation between prognostic factors such as grade, tumor size, lymph node involvement, and methylation status of this promoter. In a study conducted by Rody et al (2005), more than 2/3 of invasive tumor samples (175 invasive breast carcinomas) showed hypermethylation (Rody et al., 2005).

We observed the methylation of promoter A and ON in only a subset of tumors with absence of ER by IHC. Other researches have shown that together with aberrant methylation, histonedeacetylation is also essential for silencing of the estrogen receptor (Ferguson et al., 1995; Ferguson et al., 1997; Yang et al., 2001). However, it should be note that the genomic organization of the ER gene is significantly complex and ER expression is the result of the interaction between several promoters and their transcriptional regulators that is suggested as an indication for the contradictory findings in different researches (Kos et al., 2001). Though, ethnical differences in methylation pattern should be considered for these issues (Zhao et al., 2008).

Common immunohistochemistry categorizes protein expression as a percentage of positively stained tumor cells. In this study, estrogen receptor expression ranged from 20% to 90%, thus it is likely that tumors may comprise subclones with some quantities of ER promoter methylation. As a result, we set up a quantitative methylation assay to investigate whether these subclones might be responsible for the observed reduction of ERα expression (Johnston et al., 1995). Although, MSP is the most frequently used method in methylation detection due to its high sensitivity, it is considered as a qualitative technique and samples with both methylated and
unmethylated alleles should be further explored using methylation sensitive quantitative techniques (Pasquali et al., 2007). As Xiong et al (1997) suggested, COBRA is accurate, sensitive, reliable and quantitative methods to calculate levels of DNA methylation at specific gene loci (ZhXiong, 1997).

This study was the first which calculated the percentage of methylation at ERα promoter A in patients whose both methylathed and unmethylated allele were positive. Additionally, an analysis of the relationship between the percentage of methylation at ERα promoter A and the prognostic factors such as tumors grade, lymph node involvement and size of the tumors was performed. The percentage of DNA methylation at this site ranged from 1% to 91%. Based on COBRA we found that the percentage of DNA methylation at promoters A region highly correlated with tumors grade(p= 0.27, r=0.65; Table 6, fig.2B). Additionally, these percentages at promoters A region showed borderline associations with lymph node involvement (p=0.079, r=0.55; Table 6, Fig. 2D). In conclusion, we found that Methylation of CpG site in ERα promoter A seems to be a common event in breast tumors. More than two-thirds of all tissue tumors showed aberrant methylation patterns in this promoter. This result suggests that ERα and regulation of its expression might play a crucial role in the development of malignant breast cancers. On the other hand, our study indicate that methylation of ERβ promoter ON is not responsible for the loss of gene expression in all breast tumors. Generally, 53% of the samples demonstrated methylation of either ERα or ERβ but not both. These results suggest that aberrant methylation of these CpG islands does not indicate a generalized increase in CpG island methylation but may reflect a more particular assortment process targeting key suppressor genes.

References


