Methylation Status of Tumor-Related Genes in Mcf7, Mda-Mb-468 and Bt-474 Breast Cancer Cell Lines with Different Estrogen Receptor Status

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Abstract

Background: Many breast cancer tumor suppressor genes have been reported to undergo hypermethylation, including ER, RASSF1A, HIC, CDH1, GSPT1, APC, TWIST and CCND2. We first determined the promoter methylation of the above eight tumor suppressor genes as epigenetic markers of breast cancer in three cell lines with different ER status.

Methods: This study was an experimental study performed on three cell lines: ER-positive MCF-7, ER-negative MDA-MB-468 and the ER- and HER2-positive breast cancer cell line (BT-474). For this purpose DNA was extracted from these cell lines and gene promoter methylation was analyzed by methylation-specific PCR.

Results: Our results showed that all studied genes were hypermethylated in ER-negative MDA-MB-468 cells. We detected hypermethylation of GSPT1, RASSF1A and CCND2 genes in these three cell lines. In addition, the ER-positive MCF-7 cells showed methylation of TWIST and APC genes.

Conclusion: The numbers of methylated molecular biomarkers is increased with tumor progression. This finding may be important in breast cancer therapy for different ER status.

Keywords: Hypermethylation, Breast cancer, ER status

Introduction

Epigenetic alteration is one of the causes of breast cancer which induces a neoplastic process by aberrant transcriptional regulation of genes implicated in cellular proliferation, survival and differentiation. DNA methylation of CpG islands which is located in the upstream promoter and Exon1 region of most tumor
Suppressor genes result in transcriptional silencing in tumors and is known to be an early molecular event in carcinogenesis. Many tumor suppressor genes have been identified from breast neoplasms as predictive and prognostic biomarkers. Hypermethylated genes such as estrogen receptor α (ER), glutathion-S-transferase P1 (GSTP1), adenomatous polyposis coli (APC), ras-associated domain family 1 (RASSF1A), hypermethylated in cancer (HIC1), human basic helix-loop-helix DNA binding protein (TWIST), cyclin D2 (CCND2) and E-cadherin (CDH1) are used as hallmarks of breast cancer prognosis.

On the other hand, the ER-positive breast tumors respond to hormone therapy and the presence of ER acts as a predictive marker. It has been reported that up to one-third of breast cancers do not express ER at the time of diagnosis and even some ER-positive breast cancers lose ER during tumor progression. There are findings which suggest that ER status of breast cancer affects methylation of some breast tumor-related genes. Methylation-specific PCR (MSP)-based studies of primary breast tumors have demonstrated that ER promoter methylation has been observed in approximately 18% of Iranian female patients. To investigate the epigenetic changes of breast tumor-related genes in association with ER status, we assessed the methylation of GSTP1, APC, RASSF1A, HIC1, TWIST, CCND2 and CDH1 in ER-positive and ER-negative breast cancer cell lines.

The GSTP1 gene encodes an enzyme which detoxifies hydrophobic electrophil's and protects the body from environmental and dietary carcinogens. Increased GSTP1 methylation has been shown with tumor size and metastasis. The CDH1 and APC genes encode proteins involved in cell adhesion and their loss allows tumor cell metastasis. Loss of CDH1 expression is correlated with poor tumoral differentiation, increased invasion and decreased patient survival. Our group has previously found that CDH1 methylation was present in 41% of primary breast cancers and a correlation existed with increased tumor size and lymph node metastasis in Iranian patients. APC plays a role in the Wnt signal transduction pathway which is involved in cell proliferation and epithelial-mesenchymal transition in epithelial breast tumors. RASSF1A gene encodes a cellular growth-inhibitory signaling protein which is involved in cell cycle control and apoptosis signaling. RASSF1A hypermethylation has been observed in advanced breast tumors.

Figure 1. DNA methylation analysis of the indicated genes by MSP. U and M show amplified unmethylated and methylated bands, respectively. In vitro-methylated DNA (IVD) was used as a positive methylated control. CN represents DNAs from normal tissue. H2O is a negative control reaction without DNA.
tumor stages and is associated with poor prognosis.\textsuperscript{16,20,21} \textit{HIC1} is aberrantly hypermethylated in breast cancers.\textsuperscript{22} Promoter methylation of \textit{RASSF1A} and \textit{HIC1} have been shown to occur more frequently (51\% and 79\%, respectively) in invasive metastatic breast tumors in Iranian patients.\textsuperscript{23} \textit{TWIST1} is a transcription factor which plays an important role in cell differentiation and survival.\textsuperscript{24,25} Increased \textit{TWIST1} methylation has been observed in malignant breast cancers.\textsuperscript{26} \textit{CCND2} is involved in cell cycle regulation and its methylation has been correlated with poor prognosis.\textsuperscript{27}

In the present study we have investigated the methylation status of eight tumor suppressor gene promoters in three cell lines, one of which (MDA-MB-468 cell line) is methylated and the others (MCF-7 and BT-474 cell lines) are unmethylated for the ER promoter. We subsequently evaluated whether ER status in these cell lines was associated with epigenetic changes in these breast cancer markers.

**Materials and Methods**

**Cell culture and DNA extraction**

This was an experimental study. We obtained ER-negative MDA-MB-468, ER-positive MCF-7 and BT-474 (also HER2 positive) cells from the National Cell Bank of Iran (Pasture Institute, Iran). The human breast cancer cell lines MDA-MB-468, MCF-7 and BT-474 were grown in

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer pair sequences (5’ 3’)</th>
<th>Annealing temperature (˚C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDH1-M</td>
<td>TTAGGTGAGGGGTATCGCGT</td>
<td>57</td>
</tr>
<tr>
<td>CDH1-U</td>
<td>TAACTAAAAACCTACCTACCGAC</td>
<td>53</td>
</tr>
<tr>
<td>ESRe-M</td>
<td>TTGGGATGTTATTTTCGTC</td>
<td>59</td>
</tr>
<tr>
<td>ESRe-U</td>
<td>AAACAAATACACCCATCATCACAAC</td>
<td>59</td>
</tr>
<tr>
<td>HIC1-M</td>
<td>TGGTTTCGCGTTTTGTTGCTG</td>
<td>60</td>
</tr>
<tr>
<td>HIC1-U</td>
<td>TTGGGTTTGGTTTTGTGTTTG</td>
<td>60</td>
</tr>
<tr>
<td>RASSF1A-M</td>
<td>GGGTTTGGGAGGCAGCGC</td>
<td>64</td>
</tr>
<tr>
<td>RASSF1A-U</td>
<td>GCTAACAACGGCAACGC</td>
<td>64</td>
</tr>
<tr>
<td>CCND2-M</td>
<td>TAGTGTGTTAGGTCATCGC</td>
<td>54</td>
</tr>
<tr>
<td>CCND2-U</td>
<td>GTTATGTTATTTGTTGTATG</td>
<td>54</td>
</tr>
<tr>
<td>GSTP1-M</td>
<td>TTGGGTTGTCGCGTC</td>
<td>61</td>
</tr>
<tr>
<td>GSTP1-U</td>
<td>GCCCAATACTAAATACAGGACG</td>
<td>61</td>
</tr>
<tr>
<td>TWIST-M</td>
<td>TTGGGATGCTGGTTTTTATCG</td>
<td>61</td>
</tr>
<tr>
<td>TWIST-U</td>
<td>ACCTTCTTCAACAAACACAACACAA</td>
<td>56</td>
</tr>
<tr>
<td>APC-M</td>
<td>TATTCGAGGTCGCTG</td>
<td>60</td>
</tr>
<tr>
<td>APC-U</td>
<td>GTTGGTTTATTGTTGTTGTTG</td>
<td>60</td>
</tr>
</tbody>
</table>

M: Methylated-specific primers; U: Unmethylated-specific primers.
RPMI 1640 (Biosera, UK) supplemented with L-glutamine to 2 mM and 10% fetal calf serum (Cinnagen, Iran), after which they were sub-cultured 2-3 times in a week. There were 20 human normal breast tissue samples obtained from the Department of Pathology at Shiraz University of Medical Sciences, Shiraz, Iran. DNA was isolated from the cells by a standard phenol/chloroform procedure and frozen until analysis at -20°C.

Methylation-specific PCR (MSP)

We used the MSP method to study eight epigenetic breast cancer markers in three breast cancer cell lines. This method is the most widely used technique for studying the methylation of CpG islands, however it is non-quantitative. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which are then converted to thymidine during the subsequent PCR step, giving sequence differences between methylated and unmethylated DNA. Genomic DNA was treated with sodium bisulfate as reported by Herman et al. Genomic DNA was treated with sodium bisulfate as reported by Herman et al. Briefly, 2 µg of genomic DNA in a volume of 50 µl was denatured by NaOH (final concentration: 0.2 M) for 10-15 min at 50°C. Freshly prepared 30 µl of 10 mM hydroquinone (Fluka, UK) and 520 µl of 3 M sodium bisulfite (Fluka, UK) at pH 5 were added and mixed, after which samples were incubated at 50°C for 16 h. Modified DNA was purified using a DNA purification kit (Fermentas, UK) and eluted into 50 µl of water. Extracted DNA was treated with NaOH (final concentration, 0.3 M) for 5-10 min at room temperature. DNA was ethanol precipitated and resuspended in water. Modified DNA samples were used immediately or stored at -20°C until use. Table 1 summarizes the sequence of PCR primers used to distinguish methylated and unmethylated tumor related genes and annealing temperatures. The PCR reaction mixture contained 1x PCR buffer, dNTPs (each at 0.3 mM), and MgCl2 (3 mM) which were all provided by Fermentas, primers (0.5 mM each per reaction), and bisulfite-modified DNA (100-150 ng) or unmodified DNA (100-150 ng) in a final volume of 25 µl. Reactions were hot-started at 94°C for 5 min before the addition of 1.25 units of Taq DNA polymerase (Fermentas, UK). Amplification of two genes were performed under the following conditions: 35 cycles at 95°C for 30 s, the specific annealing temperature listed in Table 1 for 30 s, and at 72°C for 30 s; and a final extension for 4 min at 72°C. Human normal DNA samples were used as the controls for unmethylated alleles. A reaction without DNA was used as the negative control for each set of PCR reactions. In each case in vitro-methylated DNA (IVD) was used as the positive control. A total of 8 µl of each PCR reaction was loaded onto a 2% agarose gel and visualized under UV illumination. The PCR for samples was repeated three times to confirm the reproducibility of the results. All analyses were performed using the t-test by SPSS version 11.5 statistical software (Chicago, IL).

Results

Analysis of methylation distribution in MCF7, MDA-MB-468 and BT-474 cell lines

We searched for aberrant promoter methylation at ERα, GSTP1, APC, RASSF1A, HIC1, TWIST, CCND2 and CDH1 gene loci in the MCF7, MDA-MB-468 and BT-474 cell lines. These data are summarized in Table 2. Our results (Figure 1) showed that all studied genes were hypermethylated in ER-negative MDA-MB-468 cells. However, all of these sites were unmethylated in normal samples. We used 20 human normal DNA samples as controls. The controls were all unmethylated for the studied alleles. CpG islands are usually unmethylated in DNA from normal tissues. The results for control samples are presented in Figure 1. We detected hypermethylation of GSPT1, RASSF1A and CCND2 genes in the three cell lines. In addition, the ER-positive MCF-7 cells showed methylation of TWIST and APC genes. We observed CDH1 and HIC methylation in ER-negative MDA-MB-468 cells and unmethylated in ER-positive MCF7 and BT-474 cell lines.


Discussion

Estrogen receptors play an important role in the initiation, progression and prognosis of breast cancers. It has been reported that two-thirds of breast cancers express ER protein at levels higher than normal breast tissues. They are more differentiated and responsive to hormonal therapies. However one-third of all breast cancers are ER-negative, which are associated with poorer differentiation, higher growth fraction and worse clinical outcome compared with ER-positive tumors.29

Breast cancer is a disease driven by epigenetic alterations. These alterations change the expression patterns of genes implicated in cellular proliferation, survival and differentiation. Epigenetic changes occur by DNA methylation and histone modification. Tumor suppressor gene hypermethylation can lead to transcriptional silencing of these genes. Many tumor suppressor genes have been identified as epigenetic markers of breast cancer.1 Promoter methylation as a cancer marker occurs more than mutations and is shown to be an early event in tumor genesis.3 These molecular biomarkers have been reported as important targets for breast cancer diagnosis in early stage and therapy.1

We chose eight molecular biomarkers - ERα, GSTP1, APC, RASSF1A, HIC1, TWIST, CCND2 and CDH1 in the present study. We compared our results for these three cell lines with data found for methylation of these genes in ER-positive and ER-negative breast cancer tumors and cell lines. GSTP1 gene was shown to express in ER-negative rather than ER-positive breast cancer cell lines.20 This relationship was postulated to be the result of methylation-associated inactivation of the GSTP1 gene by estrogen metabolites.31 Despite this, our results showed GSTP1 to be methylated in both ER-negative and ER-positive breast cancer cell lines. We also detected RASSF1A and CCND2 methylation in ER-positive and ER-negative breast cell lines. The association of RASSF1A methylation and ER-positive breast cancer tumors was reported in previous studies.6,7,32,33 However, methylation status of CCND2, GSTP1, APC and TWIST, whose promoter methylations have been reported to be associated with ER positivity of breast cancer samples,6,7,34 failed to show correlations with ER status in other studies33 and the current study. We found differences in the methylation of CDH1 and HIC between ER-positive and ER-negative cell lines. In contrast, other studies showed no differences in methylation of CDH17,35 between ER-positive and ER-negative groups. Our previous studies in Iranian patients also showed that methylation of RASSF1A, HIC1 and CDH1 did not correlate with ER status.8,23 This discrepancy might be due to the limited number of cell lines studied. In some reports HER2 status was also shown to be correlated with epigenetic alteration.7,34 In the present study we observed differences in methylation status of TWIST and APC genes between the HER2-positive cell line (BT-474) and ER-positive cell line (MCF7). These findings showed lesser gene methylation in the HER2-positive cell line compared to the ER-positive cell line. Different studies have shown, however, that methylation of many tumor suppressor genes is not restricted to ER status. In contrast, tumor suppressor methylation occurs at high frequencies in both ER-positive and negative tumors. CpG island methylation of tumor-related genes accumulates with breast cancer progression,35 consistent with the idea that increased numbers of methylated molecular biomarkers which started from an early stage of

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>ERα</th>
<th>GSTP1</th>
<th>APC</th>
<th>RASSF1A</th>
<th>HIC1</th>
<th>TWIST</th>
<th>CCND2</th>
<th>CDH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>U</td>
<td>M</td>
<td>U+M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
</tr>
<tr>
<td>BT-474</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>U+M</td>
<td>U+M</td>
<td>M</td>
</tr>
</tbody>
</table>

U: Unmethylated; M: Methylated; U+M: Heterogenous, both methylated and unmethylated PCR products.

Table 2. Promoter methylation status of breast tumor-related genes in breast cancer cell lines.
breast cancer in either ER-positive or ER-negative tumors may not be restricted to the special tumor suppressor genes. It was also suggested that DNA hypermethylation makes step-wise transformation from normal to invasive breast cancer.36

The strong point of our study is that the MDA-MB-468 cell line which is a more progressive form of breast cancer than the other two studied cancer cell lines has shown hypermethylation of all studied epigenetic markers; however this should be researched more through the study of a large number of breast cancer cell lines.

In conclusion we demonstrated hypermethylation of all studied epigenetic markers of breast cancer in the ER-negative breast cell line, which should be additionally researched. In comparison with the other studies which reported accumulation of DNA hypermethylation in breast tumors, it could not be concluded that there was an association between many epigenetic markers of breast cancer and ER status of breast tumors. This was consistent with the idea that the number of methylated molecular biomarkers has been shown to increase with tumor progression. It seems HER2 status does not associate with promoter methylation of these epigenetic markers. This finding may be important in breast cancer therapy for different ER and HER2 status.

Acknowledgment

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Conflict of interest

No conflict of interest is declared.

References


