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Original Article

Hypermethylation of E-Cadherin and Estrogen Receptor-α Gene Promoter and Its Association with Clinicopathological Features of Breast Cancer in Iranian Patients

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Abstract

Background: Aberrant methylation of cytosine-guanine dinucleotide islands leads to inactivation of tumor suppressor genes in breast cancer. Tumor suppressor genes are unmethylated in normal tissue and often become hypermethylated during tumor formation, leading to gene silencing. We investigated the association between E-cadherin (*CDH1)* and estrogen receptor-α *(ESRα)* gene promoter methylation and major clinical and pathological features of breast cancer in Iranian women.

Methods: DNA was extracted from 67 primary breast tumors and gene promoter methylation was analyzed by methylation-specific polymerase chain reaction method.

Results: Fifty percent of the samples showed aberrant methylation in at least one of the two tested loci. We detected *CDH1* hypermethylation in 41% of invasive tumors and receptor-α gene hypermethylation in 18% of invasive tumor samples. We found no association between *CDH1* and receptor-α gene hypermethylation (P=0.45). There was a correlation between hypermethylation of *CDH1* locus and tumor size ≥5 cm (P=0.019).

Conclusion: Our data suggest that the malignant progression of human ductal and lobular breast carcinoma in Iranian women involves a heterogeneous pattern of cytosine-guanine dinucleotide island hypermethylation of the *CDH1* gene.
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Keywords ● Breast cancer ● ESRα ● CDH1 ● CpG island ● methylation

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Introduction

Breast cancer is one of the most common cancers among Iranian women.[1] The development of breast cancer may result from inherited mutations in the germ line or somatic cells and from epigenetic changes in patterns of gene expression. Epigenetic changes are the consequence of DNA methylation and histone modifications. These processes in tumors mostly result in inappropriate gene expression.[2] Aberrant DNA methylation includes hepermethylation and hypomethylation of the promoter or first exon

of cancer-related genes.[3] Hypermethylation of tumor suppressor genes results in transcriptional silencing, and hypomethylation of the corresponding sequences of the proto-oncogene might change them into activated oncogenes.[3] Methylation of DNA occurs on cytosine residues of the cytosine-guanine dinucleotide (CpG) dinucleotides,[4] clustered in the promoter area of genes,[5] by DNA methyl transferases.[6] Recent studies have focused on identifying the CpG island hypermethylation of specific genes in breast tumors.[7-9]

Many tumor suppressor genes in breast cancer have been reported to undergo hypermethylation including E-cadherin (*CDH1*) and estrogen receptor-α *(ESRα)*, which are involved in cell adhesion and signaling, respectively.[10] The *CDH1* gene encodes the transmembrane glycoprotein E-cadherin, whose main role is in maintaining cell-cell adhesion in epithelial tissues.[11] Decreased E-cadherin expression has been reported at high frequencies in infiltrating breast cancers associated with aberrant *CDH1* hypermethylation.[12] Decreased E-cadherin expression is one of the eight major breast tumor-markers in gene methylation studies.[13] *ESRα* as a nuclear receptor and its ligand, estradiol, are important in the development, progression, treatment, and outcome of breast cancer.[14] Breast cancers lacking *ESRα* rarely respond to endocrine therapy and are associated with a higher growth fraction.[15] Loss of *ESRα* gene transcription by methylation of its CpG island is the only epigenetic modification in the absence of mutation thus far reported in many human breast cancer cell lines and tumors.[16,17] Loss of expression of both *CDH1* and *ESRα* genes has been reported frequently in the initiation or progression of breast cancer.[12,18] Additionally, several studies have evaluated hypermethylation of these genes in serum to detect cancer.[19,20] Certain tumor suppressor genes undergo methylation in malignant tumors, including breast cancer.[21] Identification of a gene's methylation status can help to elucidate the molecular pathogenesis of breast cancer. It may also have clinical value for the early detection of breast cancer cells in body fluids such as mammary aspiration fluids.[22,23] Many studies have reported *CDH1* and *ESRα* methylation frequencies in breast cancers of different populations.[8,9,20] It has been suggested that the frequency of *ESRα* methylation is higher in Asians than Americans.[8] The aim of present study was to investigate the association between *CDH1* and *ESRα* gene methylation and distinctive clinical and pathological features of breast cancer in Iranian patients.

Materials and Methods

Specimens and DNA Extraction

A total of 67 human breast tumor specimens identified as ductal carcinoma *in situ* (n=1), infiltrating ductal carcinoma (n=59), infiltrating lobular carcinoma (n=6) and metaplastic carcinoma (n=1) were obtained from the Department of Pathology at Shiraz University of Medical Sciences and from the Department of Pathology at Dena Hospital in Shiraz, Iran. DNA was isolated from the tissues as described previously.[24] DNA samples were labeled with a coded identification number so that the analysis could be performed without knowledge of specimen origin.

Methylation-Specific PCR

Methylation-specific PCR is the most widely used technique for studying the methylation of CpG islands. 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, revealing sequence differences between methylated and unmethylated DNA.[25] Genomic DNA was treated with sodium bisulfate as reported by Herman *et al*.[25] Briefly, 2 µg of genomic DNA was denatured in 50 µl NaOH (final concentration, 0.2 M) for 10-15 min at 50 °C. Thirty microliters of 10 mM hydroquinone (Fluka. Corp. Milwaukee, WI, USA) and 520 µl 3 M sodium bisulfite (Fluka Corp. Milwaukee, WI, USA) at pH 5, both freshly prepared, were added and mixed, and the samples were incubated at 50 °C for 16 h. Modified DNA was purified with a DNA purification Kit (Fermentas, Inc, Ontario, Canada) 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 for further analysis.

The sequence of PCR primers used to distinguish methylated and unmethylated *CDH1* and *ESRα* genes, annealing temperatures, and expected sizes of PCR products are summarized in table 1. The *CDH1*,[26] and *ESRα*,[27] specific primers were purchased from (Cinnagen, Inc, Tehran, Iran), and we amplified a region from −205 and −210 bp upstream to +44 bp downstream from the transcription start site, respectively (figure 1A). The PCR reaction mixture contained Ix PCR buffer, dNTPs (each at 0.3 mM), $MgCl_2$ (3 mM) all provided by enzyme supplier (Fermentas, Inc, Ontario, Canada), primers (0.5 mM each per reaction), and bisulfite-modified DNA (100−150 ng)

Table 1: Methylated specific primer sequences for *CDH1* and *ESRα* genes.

Name	Primer pair sequences (5'—3')	Size (bp)	Anneal T (˚C)	Sites [a]
CDH1-M [b]	TTAGGTTAGAGGGTTATCGCGT TAACTAAAAATTCACCTACCGAC	116	57	-205
CDH1-U [c]	TAATTTTAGGTTAGAGGGTTATTGT CACAACCAATCAACAACACA	97	53	-210
ESRα-M	TTTGGGATTGTATTTGTTTTCGTC ACAAAATACAAACCGTATCCCCG	192	59	+44, +236
ESRα-M	TTTGGGATTGTATTTGTTTTTGTTG AAACAAAATACAAACCATATCCCCA	192	59	+44, +236

[a] Relative to transcription start, [b] M: methylated-specific primers, [c] U: unmethylated-specific primers

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, Inc, Ontario, Canada). Amplification of two genes was performed under the following condition: 35 cycles of 95 °C for 30 s, the specific annealing temperature for 30 s and 72 °C for 30 s, and a final extension of 4 min at 72 °C (table 1). Samples of normal human DNA were used as the control for unmethylated alleles. A reaction without DNA was used as a negative control reaction for each set of PCR reactions. Each PCR reaction was loaded (at a volume of 8 µl) onto a 2% agarose gel and visualized under UV illumination. The PCR for samples demonstrating methylation was repeated to confirm reproducibility of the results.

Statistical Analysis

The Chi-squared test was used to determine associations between the methylation of *CDH1* and *ESRα* genes and various phenotypic features of breast cancer. Methylation frequencies of two loci were compared using Fisher exact test. All analyses were performed using SPSS version 12.0 software (Chicago, IL, USA).

Results

Table 2 shows the details of associations between *CDH1* and *ESRα* gene methylation and clinical and histological parameters in Iranian patients with breast cancer. Fifty percent of breast tumor specimens showed aberrant methylation in at least one of the two tested loci. Methylation of the *CDH1* gene was observed in 37% (22 of 59) and *ESRα* methylation in 16% (11 of 59) of specimens obtained from patients with infiltrating ductal carcinoma. Methylation of both genes was present in 8% of the specimens from infiltrating ductal carcinoma, and 66% (4 of 6) of specimens from infiltrating lobular carcinoma showed *CDH1* methylation. Only one of these specimens was methylated at both loci. The metaplastic carcinoma specimen

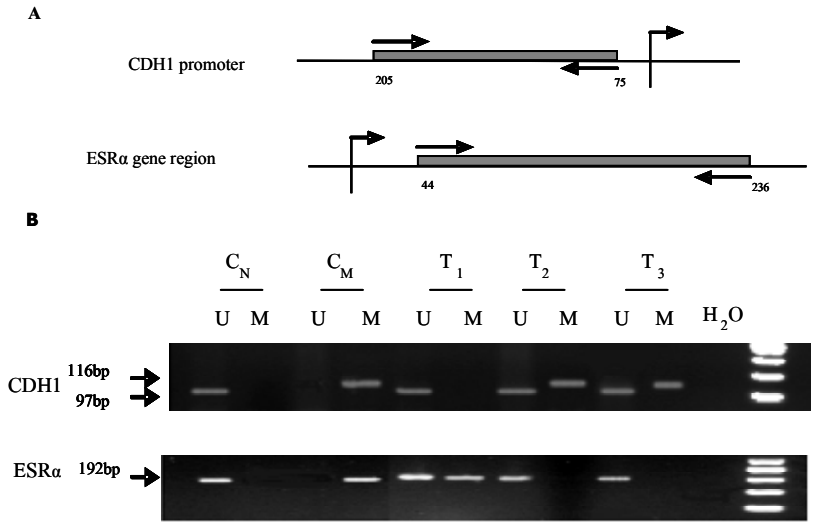

Figure 1: Methylation-specific PCR of the *CDH1* and *ESRα* genes. **A:** Location of primers designed for methylation-specific PCR of the *CDH1* and *ESRα* genes. The vertical arrows demonstrate the transcription start sites and horizontal arrows show the direction of transcription. The gray boxes show the regions of *CDH1* and *ESRα* CpG islands that were amplified. **B:** DNA methylation analysis of the *CDH1* and *ESRα* CpG islands by methylation-specific PCR. U and M represent amplified unmethylated and methylated bands, respectively. N and M represent DNAs from normal tissue and a methylated control sample, respectively. H_2O is a negative control reaction without DNA.

Table 2: Association between *CDH1* and *ESRα* gene methylation and clinicopathological features in patients with breast cancer.

Features (n) [a]		Methylation status of *ESRα* gene		P value	Methylation status of *CDH1* gene		P value
		Methylated	Unmethylated		Methylated	Unmethylated	
Total	67 (100 %)	12 (17.9 %)	55 (82.1%)		27 (40 %)	40 (60%)	
Age							
≤ 50 yrs	43 (64 %)	9 (20.9 %)	34(79.1%)	P=0.173	21 (48.5%)	22 (51.2%)	P=0.073
> 50 yrs	23 (34 %)	2 (8.7%)	21 (91.3%)		6 (26%)	17 (73.9%)	
Side							
Right	33 (53.2 %)	3 (9.1%)	30 (90.9%)	P=0.104	11 (33.3%)	22 (66.7%)	P=0.231
Left	29 (46.8 %)	7 (24.1%)	22 (75.9%)		14 (48.5%)	15 (51.7%)	
Nodal involvement							
Negative	27 (42.9 %)	4 (14.8%)	23 (85.2%)	P=0.495	9 (33.3 %)	18 (66.7%)	P=0.5
Positive	36 (57.1 %)	8 (22.2%)	28 (77.8%)		15 (41.7%)	21 (58.3%)	
Tumor size							
< 2 cm	22 (33.3 %)	4 (18.2%)	18 (81.8%)		9 (41%)	13 (59.1%)	
2-5 cm	30 (45.5 %)	6 (20%)	24 (80.8%)	P=0.9	8 (26.7%)	22 (73.3%)	P= 0.019
> 5 cm	14 (21.2 %)	2 (14.3%)	12 (85.7%)		10 (71.4%)	4 (28.6%)	
Histological type							
IDC	59 (88 %)	11 (18.6%)	48 (81.4%)		22 (37.3%)	37 (62.7%)	
ILC	6 (9 %)	1 (16.7%)	5 (83.3%)	P=0.695	4 (66.7%)	2 (33.3%)	P=0.168
Others	2 (3 %)	0	2 (100%)		1 (50%)	1 (50%)	
Histological grade							
I	7 (12 %)	2 (28.6%)	5 (71.4%)	P=0.374	2 (28.6%)	5 (71.4%)	P=0.5
>I	51(87.9 %)	8 (15.7%)	43 (84.3%)		19 (37.3%)	32 (62.7%)	
Estrogen receptor status							
Positive	46 (80.7 %)	8 (17.4%)	38 (82.6%)	P=0.164	18 (39.1%)	28 (60.9%)	P=0.170
Negative	11 (19.3 %)	4 (36.4%)	7 (63.6%)		2 (18.2%)	9 (81.8%)	
Progesterone receptor status							
Positive	36 (63.2 %)	6 (16.7%)	30 (83.3%)	P=0.232	12 (33.3%)	24 (66.7%)	P=0.716
Negative	21 (36.8 %)	6 (28.6%)	15 (71.4%)		8 (35.1%)	13 (61.9%)	

[a] Data were not available for age and tumor size in one patient, tumor side in five patients, nodal involvement in four patients, tumor grade in nine patients, and estrogen and progesterone receptor status in 10 patients. IDC: infiltrating ductal carcinoma, ILC: infiltrating lobular carcinoma

showed methylation of the *CDH1* promoter (table 2). We found that methylation of *CDH1* and *ESRα* genes was heterogeneous in invasive tumor specimens. In all of the specimens with methylation in both *CDH1* and *ESRα* genes, unmethylated alleles were also present (figure 1B). Control reactions with untreated DNA demonstrated that PCR products were obtained only from bisulfite-treated DNA, confirming that our data did not contain any false positive results caused by incomplete bisulfite treatment (data not shown).

The mean age of patients at the time of diagnosis of primary breast cancer was 47 years, ranging from 27 to 75 years. Methylation frequencies were compared with distinctive clinical features with Chi-square tests. No statistically significant differences in the frequencies of *CDH1* and *ESRα* gene promoter methylation were found according to the patients' lymph node status, histological grade and type, or estrogen and progesterone receptor status. However, there was a significant association between *CDH1* methylation and tumor size ≥5 cm (P=0.019). A borderline association was also observed between *CDH1* methylation and age of 50 years or less (P=0.073).

Discussion

Aberrant methylation appears to be an early event in the etiology of breast tissue carcinogenesis, resulting in the down regulation and silencing of cancer-related genes.[10,28] Numerous studies have reported that CpG island hypermethylation is implicated in the loss of expression of a variety of critical tumor suppressor genes and growth regulatory genes including cell cycle regulators, steroid receptors, and cell adhesion molecules in breast carcinoma. Therefore, loss of these gene products gives rise to increased susceptibility to tumor development or decreased detoxification of carcinogens.[6]

To better understand the role of epigenetic events in the etiology of breast cancer among Iranian women, we examined the methylation status of two genes in primary breast cancers and their association with distinctive pathological characteristics. In the present study we used methylation-specific PCR to detect methylated and unmethylated alleles. This detection technique is a rapid and cost-effective clinical tool used to study CpG island hypermethylation in human tumors. The high sensitivity of this method allows the methylation status of extracted DNA to be analyzed with a

vertical resolution of 0.1%.[21,25]

We found a correlation between hypermethylation at the *CDH1* locus and tumor size (P=0.019). We detected *CDH1* hypermethylation in 41% (27 of 66) of invasive tumor specimens. In contrast, *ESRα* gene hypermethylation was observed only in 18% (12 of 66) of invasive tumor specimens. It has been previously shown that a subset of tumors has different aberrant *CDH1,*[10,12,18,29] and *ESRα,*[10,20,27,29,30] gene methylations. We found that the frequency of *ESRα* methylation was lower than the numbers reported earlier in different populations (34.8% to 84%).[8,10,18,20,29,30] But *CDH1* methylation frequency was similar to the findings reported in Italy in 48% of patients with breast cancers.[10] Studies in Brazil and Australia have reported *CDH1* hypermethylation in 72% and 80% of their cases, respectively figures that are higher than in the present study.[12,29] Recently, a report suggested that the reason for different gene promoter methylation frequencies in breast cancers in various studies might be differences in ethnic and socioeconomic characteristics in each area.[8] Some previous studies revealed the coincidence of CpG island hypermethylation of both *CDH1* and *ESRα* genes in breast tumors.[10,18,29] However, we found no association between *CDH1* and *ESRα* gene hypermethylation in Iranian patients with cancer (P=0.45). This might be caused by the low frequency of *ESRα* methylation.

It has been reported that *CDH1* promoter methylation increases with breast tumor progression. Therefore, *CDH1* promoter methylation is an important event associated with the pathogenesis of breast cancer.[12,18] Consistent with these reports, we found a significant association between *CDH1* methylation and tumor size of 5 cm or more (P=0.019). Tumor size is one of the detection and prognosis criteria in breast cancer,[31] so the likelihood of methylation of the *CDH1* CpG island may increase with cancer progression. The *CDH1* gene product E-cadherin mediates cell–cell adhesion and sends signals that regulate basic cellular processes such as migration and proliferation.[32] In the cell junctions, E-cadherin binds to β-catenin and prevents translocation of this transcription factor to nucleus. Hypermethylation of the *CDH1* promoter often causes down regulation of its expression.[33] A loss of cadherin, which compromises cells' adhesive function, allows tumor cell metastasis, while translocation of β-catenin into the nucleus can induce the expression of genes that promote cell proliferation and invasion.[32,33] It has been shown that the methylation of *CDH1* is associated with younger age.[29] Our analysis also indicated that the methylation pattern of the *CDH1* gene was slightly (P=0.073) correlated with patients' age, suggesting that *CDH1* methylation occurs early, prior to invasion. Early *CDH1* methylation in the beginning stages of tumor genesis may trigger the genetic events leading to tumor development at a lower age. This epigenetic change for breast tumors can be important for the early diagnosis and prevention of cancer. Nevertheless, these findings should be confirmed in other studies.

Consistent with a previous report,[18] our data indicated that CpG island methylation was heterogeneous in breast cancer. In all the specimens with *CDH1* and *ESRα* gene methylation, unmethylated alleles were also present (fig 1B). However, the presence of unmethylated alleles might be caused by a mixture of normal and tumoral cells.

CpG island hypermethylation of the *CDH1* gene and other tumorigenesis factors might be used as a biological marker for cancer cell detection. It can also be used to detect and predict breast cancer development in nipple ductal lavage fluids and needle aspirates of the breast, as reported for methylation analysis of *cyclin D2*, *RARβ*, *Twist*, *GSTP1*, *p16*, *p14*, *RASSF1A* and *DAPK* genes in mammary aspirates.[22,23]

We found no correlation between *ESRα* methylation and estrogen receptor status. In agreement with previous reports,[20] because of heterogonous *ESRα* methylation, the estrogen receptor protein was present in detectable levels. In estrogen receptor-negative specimens with no *ESRα* methylation, other mechanisms including histone deacetylation,[20] loss of transcriptional activators,[34] and chromatin remodeling,[35] are possibly involved in estrogen receptor silencing.

In conclusion, our data indicated that the malignant progression of human ductal and lobular breast carcinoma in Iranian patients involves a heterogeneous pattern of CpG island hypermethylation in the *CDH1* gene. Particularly, loss of *CDH1* expression is associated with the acquisition of invasion, and metastatic potential.

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Conflict of Interest: None declared

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