

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

Hora Loghmani¹, Mehrdad Noruzinia^{1*}, Hossein Abdul-Tehrani¹, Mahdieh Taghizadeh¹, Mohammad Hamid Karbassian²

1. Department of medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

2. Department of medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

3. Department of surgery, Atieh Hospital, Tehran, Iran

Original Submission: November 2013 Revised Submission: December 2013 Accepted: January 2014

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 (ER α and ER β). 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 ER α methylation occurs in high frequency and may be one of the important mechanisms for ER α gene silencing in a subset of Iranian primary sporadic breast cancers. In the other hand, the CpG Island methylation status of ER β and the relationship between clinicopathological features and the pattern of ER β 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: ER α promoter methylation was identified in 29(72.5%) tissue samples and 35(87.5%) peripheral blood. Among these ER α -methylated cases, the co-occurrent methylation of ER promoter in peripheral blood and tissue samples was evident in 25 (71.4%) patient (P=0.56). Furthermore, ER β promoter methylation was detected in 13(32.5%) tissue samples and 4(10.0%) peripheral blood specimens. Of these ER α -methylated cases, the co-occurrent methylation of ER β 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 *Bst*UI restriction site of the ER α 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 ER α promoter and ER β 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 ER β 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

*Corresponding author: Dr. Mehrdad Noruzinia, Department of medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Tel., +9821-82884579; Email, noruzinia@modares.ac.ir

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 ER α and ER β are expressed 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 ER α 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 ER β may act as a tumor-suppressor gene. A number of studies in humans demonstrate that ER β 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 ER α 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 ER α 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 ER α (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 ER β promoter ON is highly methylated in breast cancer cell lines and DNA methylation is an important mechanism for ER β 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 mM dNTP, 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 stop 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), 1µl (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, 2µl 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 50s, 72°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 *Bst*UI, 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. *Bst*UI normally digests CGCG site when methylated and can thus differentiate the two alleles. In this reaction, *Bst*UI 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

Promoter	speciation	primer name	primer sequence	Anealing Temperature	
ERα Promoter A	Methylation Specific	ERαAMF	5'-GATACGGTTTGTATTTTGTTCGC-3'	58°C	
		ERαAMR	5'-CGAACGATTCAAAAACCTCCAAC-3'		
	UnMethylation Specific	ERαAUF	5'-GGATATGGTTTGTATTTTGTTTGT-3'	58.7°C	
		ERαAUR	5'-ACAAACAATTCAAAAACCTCCAAC-3'		
	COBRA assay		ERαCoF	5'-GGTTTTGAGTTTTTTGTTTG-3'	50°C
			ERαCoR	5'-AACTTACTACTATCCAAATACACCTC-3'	
ERβ Promoter ON	Methylation Specific	ERβMF	5'-GAGGGATTATTCGAGTTGC-3'	61°C	
		ERβMR	5'-AAATACGAACACGTACTTTTCC-3'		
	UnMethylation Specific	ERβUF	5'-GAGGGATTATTTGAGTTGT-3'	54.7°C	
		ERβUR	5'-AAATACAAACACATACTTTTCC-3'		

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 ER α promoter A and 38.23% at ER β promoter ON with MSP assay.

Analysis of CpG Island methylation status of ER α and ER β 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 2 Demographic and clinicopathological findings

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

Methylation of ERs

Methylation of ER α promoter A was observed in 35/40 (87.5%) of peripheral blood specimens whereas 13/40 (32.5%) of ER α promoter A was unmethylated in these samples. In the corresponding 34 tumor tissue samples, 29/34 (85.3%) of ER α promoter A was methylated, and 17/34 (50%) of them were unmethylated.

Furthermore, Methylation of ER β promoter ON was not observed in the peripheral blood specimens, whereas 4/40 (10%) of ER β promoter ON are methylated in these samples. In tumor tissue samples, 13/34 (38.2%) of ER β promoter ON was methylated. However in 100% (34/34) of samples the unmethylated allele was detected.

To determine if there is an association between ER α and ER β promoter methylation patterns, we analysed the methylation status of the both promoter in the 34 tumor breast tissues. ER β promoter methylation was identified in 13 tumors (38.2%). Among these 13 ER β -methylated cases, 12 cases had also ER promoter-methylation. Also, it was founded that DNA methylation level at ER α

promoter A was 2.30 folds higher than that of promoter ON in breast tissue tumors. The relationship between ER α methylation and ER β 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. ER α promoter methylation was identified in 29(72.5%) tissue samples and 35(87.5%) peripheral blood. Among these ER α -methylated cases, the co-occurrence of ER promoter methylation in peripheral blood and tissue samples was evident in 25 (71.4%) patient. Furthermore, ER β promoter methylation was detected in 13(32.5%) tissue samples and 4(10.0%) peripheral blood specimens. Of these ER α -methylated cases, the Co-occurrent methylation of ER β 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 3 Correlation between ER α and ER β promoter methylation patterns

Promoters ER β	ER α Unmethylated (%)	ER α Methylated (%)	Total
Unmethylated	4(80)	17(58.6)	21(61.8)
Methylated	1(20)	12(41.4)	13(38.2)
Total	5(14.7)	29(85.3)	34(100.0)

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

Table 4 Relationship between ER α Methylation in peripheral blood and tissue samples. Pearson Chi-Square test; P = 0.56

Promoters ER α /Blood	No tissue (%)	ER α Unmethylated /Tissue (%)	ER α Methylated/ Tissue (%)	Total
Unmethylated	0(0)	1(20)	4(13.8)	5(12.5)
Methylated	6(100)	4(80)	25(71.4)	35(87.5)
Total	6(15.0)	5(12.5)	29(72.5)	40(100)

Table 5 Relationship between ERβ Methylation at promoter ON in peripheral blood and tissue samples. Pearson Chi-Square test; P = 0.11

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

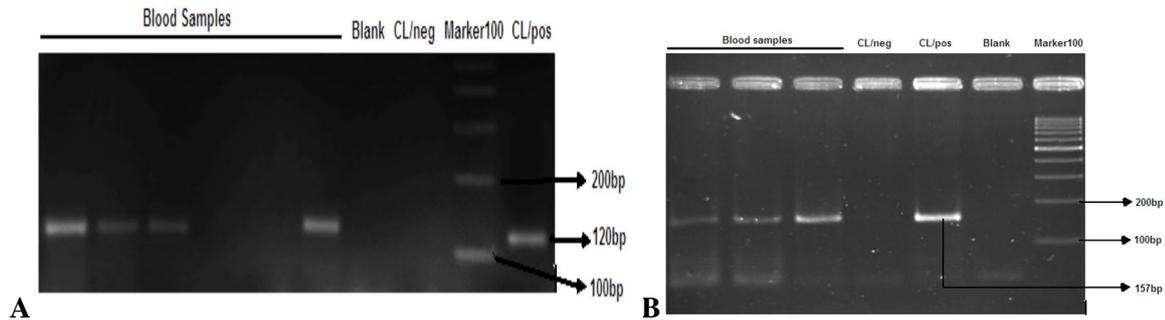


Figure 1 MSP analysis of ERα promoter A (A) and ERβ promoter ON(B) using Unmethylated and methylated sequence-specific primers in the peripheral blood samples. The products were separated by polyacrylamide gel electrophores. **A**, from right: positive control (CL/pos; Sss1 treated DNA), 100bp marker, 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 *Bst*UI, 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 *Bst*UI-cleaved PCR product and the total amount of PCR product was employed to determine the percentage of fully methylated *Bst*UI 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).

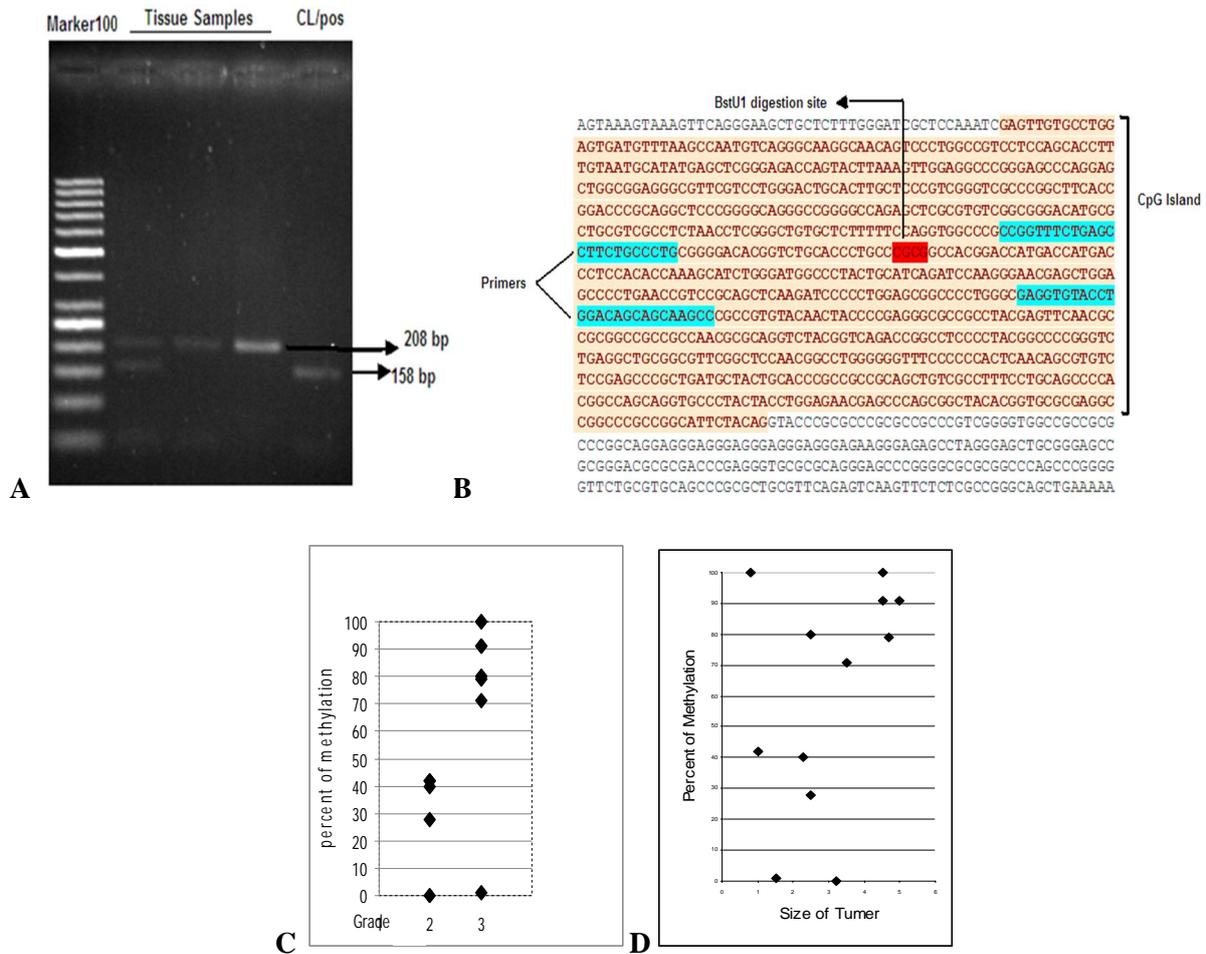


Figure 2 COBRA for analyzing the ER α promoter A methylation status. A, PCR-amplified A promoter fragment from a representative patient. The digested products were separated by polyacrylamide gel electrophoresis and enzymatic digestion performed by *Bst*UI, on the 208bp PCR product. From left: 100bp size marker, 3 tissue samples showing both the digested and undigested bands (methylated and unmethylated allele respectively), *Sss*I treated positive control (completely digested, 158bp). B, Sequence of A CpG island amplified COBRA. *Bst*UI 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 *Bst*UI sites at promoter A and clinicopathological finding

		Lymph node involvement	Grade tumors	Size of the tumors
Percentage ofMethylation	Correlation	0.55	0.65	0.52
	P-Value	0.07	0.02	0.1

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 ER α promoter A and ER β promoter ON and their association with pathological findings.

In this study, we demonstrated that methylation in ER α 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 ER α 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 ER α promoter is greater than that of many other genes (Kos et al., 2001) and various ER- α 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 ER α , ER β has a complicated 5' region, with two distinct promoters, named OK and ON. The function of ER β in breast tissue was

not completely determined. However more recent observations suggest the substantial role of ER β as a tumor-suppressor gene (Rody et al., 2005). In this study, we demonstrated that methylation in ER β 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, histone deacetylation 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 noted 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 ER α 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 ER α promoter A in patients whose both methylated and unmethylated allele were positive. Additionally, an analysis of the relationship between the percentage of methylation at ER α 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. 2 B). Additionally, these percentages at promoters A region showed borderline associations with lymph node involvement ($p=0.079$, $r=0.55$; Table 6, Fig. 2D).

Widschwendter et al (2008) found that factors like estrogens that can be modulated by the environment leave an imprint on the DNA of cells that are unrelated to the mammary breast tissue and indicate the predisposition to develop a cancer (Widschwendter et al., 2008). In our study the frequency of methylation at ER β in tissue was approximately 2.5 times higher than that of peripheral white blood cells, and the same methylation frequency at ER α promoter A was observed in tumors and peripheral white blood cells. But we found no significant correlation between the methylation status of ER promoters in tissue tumors and peripheral white blood cells of the patients (Table 4 and 5).

In this study, the average age of all the patients was 50.52 ± 7.69 years, and consistent with the findings in other studies (Ramezani et al., 2012; Mousavi et al., 2006); it was confirmed that the onset age of breast cancer is

a decade earlier in Iranian women compared with western counterparts (Mousavi et al., 2007; Kolahdoozan et al., 2010).

In conclusion, we found that Methylation of CpG site in ER α 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 ER α 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 ER β promoter ON is not responsible for the loss of gene expression in all breast tumors. Generally, 53% of the samples demonstrated methylation of either ER α or ER β 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

1. A Rody, U Holtrich, C Solbach, K Kourtis, G von Minckwitz, et al., 2005. Methylation of estrogen receptor b promoter correlates with loss of ER-b expression in mammary carcinoma and is an early indication marker in premalignant lesions *Endocrine-Related Cancer* *EndocrRelat Cancer*, 2(4): p. 903-16.
2. Allred DC, Harvey JM, Berardo M, Clark GM., 1998. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol*, 11: 155-68.
3. Bardin A, Boulle N, Lazennec G, Vignon F and Pujol P, 2004. Loss of ER beta expression as a common step in estrogen-dependent tumor progression. *Endocrine-Related Cancer* 11 537-551.
4. Buyru N, Altinisik J, Ozdemir F, et al., 2009. Methylation profiles in breast cancer. *Cancer Invest*, 27, 307-12.
5. F Ramezani, S Salami, M.D Omrani, D Maleki, 2012. CpG Island Methylation Profile of Estrogen Receptor Alpha in Iranian Females with Triple Negative or Non-triple Negative Breast Cancer: New Marker of Poor Prognosis, *Asian Pacific J Cancer Prev*, 13, 451-457

6. Fabianowska-Majewska K, Kordek R, Krawczyk B., 2006. Studies on the methylation status of CpG sequences located in promoters of selected tumour suppressor genes in breast cancer cells. *Nucleosides Nucleotides Nucleic Acids*, 25:1025–1028.
7. Ferguson AT, Lapidus R, Baylin S, Davidson NE., 1995. Demethylation of the estrogen receptor gene in estrogen receptor negative breast cancer cells can reactivate estrogen receptor gene expression. *Cancer Res*, 55:2279–83.
8. Ferguson AT, Lapidus RG, Baylin SB, Davidson NE., 1995. Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. *Cancer Res*, 55:2279–2283.
9. Ferguson AT, Vertino P, Spitzner J, Baylin SB, Muller M, et al., 1997.. Role of estrogen receptor gene demethylation and DNA-methyltransferase-DNA adduct formation in 5-aza-2'-cytotoxicity in human breast cancer cells. *J BiolChem*, 272:32260-6.
10. Graff, J. G., Herman, J. G., Lapidus, R. G., E-Chopra, H., Xu, R., et al., 1995. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinoma. *Cancer Res.*, 55: 5195-5199.
11. Hanstein B, Djahansouzi S, Dall P, et al., 2004. Insights into the molecular biology of the estrogen receptor define novel therapeutic targets for breast cancer. *Eur J Endocrinol* 150:243–255.
12. Harirchi I, Karbakhsh M, Kashefi A, Momtahan AJ, 2004 .Breastcancer in Iran: results of a multi-center study .*Asian Pac J Cancer Prev*. 5(1):24-7.
13. Hayashi S, Imai K, Suga K, Kurihara T, Higashi Y, et al., 1997. Two promoters in expression of estrogen receptor messenger RNA in human breast cancer. *Carcinogenesis*, 18(3):459–464.
14. Hori M, Iwasaki M, Yoshimi F, Asato Y, Itabashi M., 1999. Hypermethylation of the Estrogen Receptor Alpha Gene Is Not Related to Lack of Receptor Protein in Human Breast Cancer. *Breast Cancer*, 6:79–86.
15. Iwase H, Omoto Y, Iwata H, et al., 1999. DNA methylation analysis at distal and proximal promoter regions of the oestrogen receptor gene in breast cancers. *Br J Cancer*, 80:1982–1986.
16. Johnston SR, Saccani-Jotti G, Smith IE, et al., 1995. Changes in estrogen receptor, progesterone receptor and pS2 expression in tamoxifen-resistant breast cancer. *Cancer Res*, 55:3331-8.
17. Khazamipour N, Noruzinia M, Fatehmanesh P, Keyhanee M, Pujol P. MTHFR promoter hypermethylation in testicular biopsies of patients with non-obstructive azoospermia: the role of epigenetics in male infertility. *Hum Reprod*. 2009;24(9):2361-4.
18. Kolahdoozan S, Sadjadi A, Radmard AR, et al., 2010. Five common cancers in Iran. *Arch Iranian Med*, 13, 143-145
19. Kos M, Reid G, Denger S, et al., 2001. Minireview: genomic organization of the human ERalpha gene promoter region. *MolecEndocrinol*, 15, 2057-63.
20. Koš M, Reid G, Denger S, Gannon F., 2001. Minireview: genomic organization of the human ERα gene promoter region. *MolEndocrinol*, 15(12):2057–2063.
21. Kuiper GG, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson JA, 1996. Cloning of a novel receptor expressed in rat prostate and ovary. *ProcNatlAcadSci USA* 93:5925–5930.
22. Lapidus RG, Ferguson AT, Ottaviano YL, et al., 1996. Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. *Clin Cancer Res*, 2:805–810.
23. Lapidus RG, Nass SJ, Butash KA, et al., 1998. Mapping of ER gene CpG island methylation-specific polymerase chain reaction. *Cancer Res*, 58:2515–2519.
24. Lee JS, Fackler MJ, Teo WW, et al., 2008. Quantitative promoter hypermethylation profiles of ductal carcinoma in situ in North American and Korean women: Potential applications for diagnosis. *Cancer BiolTher*, 7, 1398-406.
25. Li, L.C. and R. Dahiya, 2002. MethPrimer: designing primers for methylation PCRs. *Bioinformatics*, 18(11): p. 1427-31.
26. M Widschwendter1, S Apostolidou, E Raum, D Rothenbacher, H Fiegl, et al., 2008. Epigenotyping in Peripheral Blood Cell DNA

- and Breast Cancer Risk: A Proof of Principle Study. *PLoS ONE.*, 3(7): e2656.
27. Manoharan, M., et al., 2007. Epigenetic targets in the diagnosis and treatment of prostate cancer. *IntBraz J Urol*, 33(1): p. 11-8.
 28. Mehrotra J, Vali M, McVeigh M, et al (2004). Very high frequency of hypermethylated genes in breast cancer metastasis to the bone, brain, and lung. *Clin Cancer Res*, 10, 3104-9.
 29. Merkel DE, Osborne CK., 1989. Prognostic factors in breast cancer. *HematolOncolClin North Am*, 3: 641–52.
 30. Mirza S, Sharma G, Prasad CP, et al., 2007. Promoter hypermethylation of TMS1, BRCA1, ERalpha and PRB in serum and tumor DNA of invasive ductal breast carcinoma patients. *Life Sci*, 81, 280-7.
 31. Mousavi SM, Mohagheghi MA, Mousavi-Jerrahi A, et al., 2006. Burden of breast cancer in Iran: a study of the Tehran population based cancer registry. *Asian Pac J Cancer Prev*, 7, 571-4.
 32. Mousavi SM, Montazeri A, Mohagheghi MA, et al., 2007. Breast cancer in Iran: an epidemiological review. *Breast J*, 13, 383-91.
 33. Mousavi SM, Montazeri A, Mohagheghi MA, MousaviJarrahi A, Harirchi I, et al., 2007. Breast cancer in Iran: an epidemiological review. *Breast J.*, 13:383–391.
 34. Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, et al., 1994. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res*, 54:2552–2555
 35. Parrella P, Poeta ML, Gallo AP, et al., 2004. Nonrandom distribution of aberrant promoter methylation of cancer-related genes in sporadic breast tumors. *Clin Cancer Res*, **10**, 5349-54.
 36. Pasquali, L., et al., 2007. Quantification of CpG island methylation in progressive breast lesions from normal to invasive carcinoma. *Cancer Lett*, 257(1): p. 136-44.
 37. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, et al., 2000. Molecular portraits of human breast tumours. *Nature* 406:747–752
 38. Pujol P, This P, Noruzinia M, Stoppa-Lyonnet D, Maudelonde T., 2004. [Are the hereditary forms of BRCA1 and BRCA2 breast cancer sensitive to estrogens?]. *Bull Cancer.*, 91(7-8):583-91.
 39. Roger P, Sahla ME, Makela S, Gustafsson JA, Baldet P, et al., 2001. Decreased expression of estrogen receptor α protein in proliferative preinvasive mammary tumors. *Cancer Res* 61:2537–2541
 40. Skliris GP, Munot K, Bell SM, Carder PJ, Lane S, et al., 2003. Reduced expression of oestrogen receptor beta in invasive breast cancer and its re-expression using DNA methyl transferase inhibitor in a cell line model. *Journal of Pathology* 201 213–220.
 41. Thushangi N. Pathiraja, Vered Stearns, Steffi Oesterreich, 2010. Epigenetic Regulation in Estrogen Receptor Positive Breast Cancer—Role in Treatment Response *J Mammary Gland BiolNeoplasia*. 15(1): 35–47.
 42. Ushijima T., 2005. Detection and interpretation of altered methylation patterns in cancer cells. *Nat Rev Cancer*, 5:223–231.
 43. Wei M, Xu J, Dignam J, et al., 2008. Estrogen receptor alpha, BRCA1, and FANCF promoter methylation occur in distinct subsets of sporadic breast cancers. *Breast Cancer Res Treat*, 111, 113-20
 44. Xue, Q., et al., 2007. Promoter methylation regulates estrogen receptor 2 in human endometrium and endometriosis. *BiolReprod*, **77**(4): p. 681-7.
 45. Yan L, Yang X, Davidson NE., 2001. Role of DNA methylation and histone acetylation in steroid receptor expression in breast cancer. *J Mammary Gland BiolNeoplasia*, 6:183–192.
 46. Yang X, Phillips DL, Ferguson AT, et al., 2001. Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. *Cancer Res*, 61:7025–7029.
 47. Yoshida T, Eguchi H, Nakachi K, et al., 2000. Distinct mechanisms of loss of estrogen receptor alpha gene expression in human breast cancer: methylation of the gene and alteration of transacting factors. *Carcinogenesis* , 21:2193–2201.
 48. Zhao C, W-F Lam E, Sunter A, Enmark E, Tamburo De Bella M, et al., 2003. Expression of estrogen receptor b isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. *Oncogene* 22 7600–7606.

49. Zhao L, Wang L, Jin F, et al., 2008. Silencing of estrogen receptor alpha (ERalpha) gene by promoter hypermethylation is a frequent event in Chinese women with sporadic breast cancer. *Breast Cancer Res Treat*, 117, 253-9.
50. ZhXiong, P.W. 1997. Laird COBRA: a sensitive and quantitative DNA methylation assay 2532–2534 *Nucleic Acids Research*, Vol. 25, No. 12