

## RESEARCH COMMUNICATION

# 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

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### Abstract

One decade early onset of the breast cancer in Iranian females was reported but the basis of the observed difference has remained unclear and difference in gene silencing by epigenetic processes is suggested. Hence, this study was sought to map the methylation status of estrogen receptor (ER) gene CpG islands and its impact on clinicopathological factors of triple negative and non-triple negative ductal cell carcinoma of the breast in Iranian females. Surgically resected formalin-fixed paraffin-embedded breast tissues from sixty Iranian women with confirmed invasive ductal carcinoma were assessed by methylation-specific PCR using primer sets encompassing some of the 29 CpGs across the ER gene CpG island. The estrogen and progesterone receptors, Her-2<sup>+</sup> overexpression, and nuclear accumulation of P53 were examined using immunohistochemistry (IHC). Methylated ER3, ER4, and ER5 were found in 41.7, 11.3, and 43.3% of the samples, respectively. Significantly higher methylation of ER4 was found in the tumors with nuclear accumulation of P53, and significantly higher methylation of ER5 was found in patients with lymph node involvement and tumor with bigger size or higher grades. Furthermore, significantly higher rate of ER5 methylation was found in patients with Her-2<sup>+</sup> tumors and in postmenopausal patients with ER<sup>-</sup>, PgR<sup>-</sup>, or ER<sup>-</sup>/PgR<sup>-</sup> tumors. However, no significant difference in ERs methylation status was found between triple negative and non-triple negative tumors in pre- and postmenopausal patients. Findings revealed that aberrant hypermethylation of the ER-alpha gene frequently occurs in Iranian women with invasive ductal cell carcinoma of the breast. However, methylation of different CpG islands produced a diverse impact on the prognosis of breast cancer, and ER5 was found to be the most frequently methylated region in the Iranian women, and could serve as a marker of poor prognosis.

**Keywords:** Estrogen receptor alpha - CpG islands methylation - triple negative phenotype - breast cancer

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### Introduction

Breast cancer with 31.4 cancer age-standardized rates (ASR) and 23.2 crude incidence rate per 100,000 women is by far the most frequent cancer among Iranian women (Sadjadi et al., 2005; Mousavi et al., 2007a; Mohagheghi et al., 2009). Although the rate of breast cancer in Iran is less than well developed countries but more than 36% of the tumors occur in women under the age of 40 years and breast cancer is a high burden in the community (Mousavi et al. 2006). The most common histological type of breast cancer in Iran, like as other countries, is Infiltrating ductal cell carcinoma (IDCC)(Mousavi et al., 2008).

Estrogen and its receptors (ERs) are regulators of crucial breast epithelial cell homeostasis, such as proliferation, differentiation, and apoptosis. Mammals mainly express two ERs, ER- $\alpha$  and ER- $\beta$ , which show distinct tissue distributions and functions (Ascenzi et al., 2006; Riggins et al., 2007). Depletion of endogenous estrogen, interference with ligand-receptor interactions,

or destruction of the ER- $\alpha$  are the mechanisms in which current adjuvant hormonal therapies such as tamoxifen therapy, target the ER- $\alpha$  pathway (Salami & Karami-Tehrani, 2003). Therefore, measurement of ER and progesterone receptors (PgR) has been strongly recommended for every primary invasive breast cancer by the American Society of Clinical Oncology (ASCO)(Harris et al., 2007). Based on results of immunohistochemistry, triple negative breast cancer is a term that has been used to define a biologically diverse group of breast tumors that lack expression of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (Her-2) and represents about 15% of all breast cancers (Seal & Chia, 2010). Since, they are a particularly difficult to treat and biologically aggressive disease with limited treatment options, optimization of chemotherapy regimens may be key in treating triple negative breast cancer (Conte & Guarneri, 2009). The impact of triple negative phenotype in prognosis of Iranian females with breast cancer has been reported recently (Salami et al.,

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Different mechanisms of ER- $\alpha$  gene repression and its clinical significance in breast cancer have been investigated (Parl 2003). However, the molecular mechanisms that regulate the ER- $\alpha$  gene expression are very complex and not fully understood (Macaluso et al., 2003). Loss of ER- $\alpha$  during disease progression has been suggested as a potential mechanism of hormone resistance in breast cancer, and it may be acquired at the gene transcription or protein modification levels (Pinzone et al., 2004; Le Romancer et al., 2010). DNA methylation, covalent addition of a methyl group to DNA, is a reversible epigenetic modification which usually occurs in the CpG islands located in or near the promoter of >70% of all genes. Transcriptional silencing of the discrete genes, such as ER- $\alpha$  by the CpG island methylation could be achieved either through direct effects or via a change in chromatin conformation (Yoshida et al., 2000; Szyf et al., 2004). As a result of the investigations carried out by Lapidus et al (1998), mapping of methylation status of ER- $\alpha$  has become possible by using primer sets encompassing 29 CpGs across the ER gene CpG island. Furthermore, aberrant promoter methylation of cancer-related genes, such as ER- $\alpha$  in sporadic breast tumors and their nonrandom distributions has also been investigated (Parrella et al., 2004). The different frequencies of ER- $\alpha$  gene silencing via promoter hypermethylation have been reported in Chinese (Zhao et al., 2008), Turkish (Buyru et al., 2009), North American and Korean (Lee et al., 2008), American (Wei et al., 2008), and Indian (Mirza et al., 2007) women with breast cancer, thus revealing the diverse impact on the prognosis of cancer. As far as the methylation status of ER- $\alpha$  has not been mapped in triple negative tumors and its impact has not been compared with non-triple negative tumors, current study was sought to evaluate the hypermethylation pattern of CpGs across the ER gene CpG island in Iranian females with triple negative and non-triple negative breast tumors and impact of methylation profile in the clinicopathological features.

## Materials and Methods

### *Tissue samples and study design*

Surgically resected formalin-fixed paraffin-embedded breast tissues from 60 women with confirmed invasive ductal carcinoma were enrolled in the current study that was approved by the institutional review board and board of medical ethics. At least four slides of each tumor were stained using Hematoxylin and Eosin (HE) staining protocol, and all tumors with clear-cut diagnosis of invasive ductal carcinoma were independently selected by two expert pathologists.

The clinicopathological features of the patients were collected by retrospective review of medical records. The samples were divided into premenopausal (>50 years old) and postmenopausal ( $\geq$ 50 years old) groups. Methylation status of CpG islands and relationship with prognostic markers were assessed in each group separately.

### *Immunohistochemical (IHC) analysis*

Three slides of 5- $\mu$ m thick sections were stained for

each sample, and scoring was performed by two different expert observers, independently.

The level of nuclear p53 protein, Her-2 overexpression, and hormone receptors were evaluated in the tissue samples using Immunohistochemical (IHC) analysis. ER and PgR were stained using 1D5 and PgR636 clone antibodies, respectively (Dako, Denmark). AO485, a universally approved antibody, was used for the immunostaining of HER-2 (Dako, Denmark). For p53, monoclonal mouse anti-human p53 protein, DO-7, antibody was used, which recognizes an epitope at the N-terminal of the human p53 protein and reacts with the wild-type as well as the mutant-type p53 protein (Dako, Denmark). In each series, a nonprimary antibody incubated slide was also used as the negative control.

Allred scoring method and cutoff was used as a clinically validated scoring method for both ER and PgR. Cases were considered positive even if  $\geq$ 1% of tumor cells were stained. The Her-2 score was based on a 0–2 scale. Completely negative or faint perceptible staining of <10% of the tumor cell membranes was assigned as 0 or negative. Weak–moderate staining of the entire tumor cell membranes in >10% of the tumor cells was scored as 1+ or suspicious. Strong circumferential staining of the entire tumor cell membranes, creating a fishnet pattern in >10% of the tumor cells was considered as 2+ or positive. For p53 protein, the tissue samples were scored on the basis of the intensity of the specific nuclear staining with 10% nuclear staining as the cutoff point.

### *Tumor grading*

Based on equal importance of the three tumor features, i.e. tubule formation, nuclear pleomorphism, and mitotic count, the malignancy grade of the tumor samples was determined by modified version of the Bloom and Richardson grading system, and three prognostic categories were assigned as follows: low risk (I), intermediate risk (II), and high risk (III).

### *DNA extraction and bisulfite modification*

DNA was isolated from formalin-fixed paraffin-embedded breast tissues using QIAamp® DNA FFPE Tissue kit (Qiagen, Germany). Briefly, up to eight freshly cut sections with a thickness of up to 10  $\mu$ m and a surface area of up to 250 mm<sup>2</sup> were combined in one preparation, and paraffin was dissolved by xylene and removed by 2-min centrifugation at room temperature. The residual xylene was extracted from the sample by ethanol and subsequent full-speed centrifugation at room temperature.

Any residual ethanol was removed carefully using a fine pipette tip, and the open tubes were incubated at room temperature (15–25°C) or at 37°C, until all the residual ethanol has evaporated. The pellets were resuspended in the supplied buffer with proteinase K and incubated at 56°C for 1 h. The formaldehyde modification of nucleic acids was partially reversed using incubation at 90°C in the supplied buffer. The entire lysates were transferred to the QIAamp MinElute column carefully and DNA was eluted using elution buffer after several washing–spinning steps. The DNA yield and its purity was checked using A260/280 absorption rate and agarose gel electrophoresis.

**Table 1. Sequences of Oligonucleotides, Position and Size of PCR Product**

Primer pair sequence	Site	Size (bp)	CpG Name
5' - GGATATGGTTTGTATTTTGTGTTTGT - 3' 5' - ACAAACAATTCAAAAACCTCCAAC - 3'	+225	120	uER3
5' - GATACGGTTTGTATTTTGTTCGC - 3' 5' - CGAACGATTCAAAAACCTCCAAC - 3'	+345	130	mER3
5' - ATGAGTTGGAGTTTTTGAATTGTTT - 3' 5' - ATAAACCTACACATTAACAACAACCA - 3'	+310	151	uER4
5' - CGAGTTGGAGTTTTTGAATCGTTC - 3' 5' - CTACGCGTTAACGACGACCG - 3'	+468	158	mER4
5' - GGTGATTTGGATAGTAGTAAGTTTGT - 3' 5' - CCATAAAAAAAAAACCAATCTAACCA - 3'	+375	120	uER5
5' - GTGTATTTGGATAGTAGTAAGTTTCGTC - 3' 5' - CGTAAAAAAAAACCGATCTAACCG - 3'	+495	118	mER5

#### Methylation-specific PCR (MSP)

DNA (1 mg) was bisulfite modified using EpiTect Bisulfite Kit (Qiagen, Germany) and stored at  $-20^{\circ}\text{C}$ . Briefly, DNA solution, RNase-free water, Bisulfite Mix, and DNA protect buffer were mixed and the bisulfite DNA conversion was performed using a thermal cycler, according to the program provided in the kit insert.

We selected less studied, but most significantly methylated loci, i.e. ER3, ER4, and ER5 for methylation-specific PCR (MSP) from previously designed primer sets encompassing several CpGs across the ER gene CpG island (Lapidus et al., 1998). The sequence of the primer sets and PCR reactions are summarized in Table 1.

The total reaction was run on a 3% TAE/agarose gel, stained with ethidium bromide, and visualized by UV light. SssI methyltransferase treated DNA from the lymphocytes of healthy donors was subjected to bisulfite modification and used as a positive control. In the negative controls,  $\text{H}_2\text{O}$  was used as the template.

#### Methylation score

Methylation of each sample was scored based on the number of methylated ERs, and thus, score 0 indicated that no methylated ER was found and 3 indicated that all ERs were methylated.

#### Statistical methods

The impact of the methylation status of CpG islands across the ER gene on prognostic factors, such as tumor size and side, malignancy grade, lymph node involvement, hormone receptors, HER-2 status, and nuclear accumulation of P53 in pre- and postmenopausal patients were tested by means of valid statistical tests and  $p < 0.05$  was considered as statistically significant.

## Results

#### Demographic and clinicopathological findings

Breast tumors from females without any socioeconomic

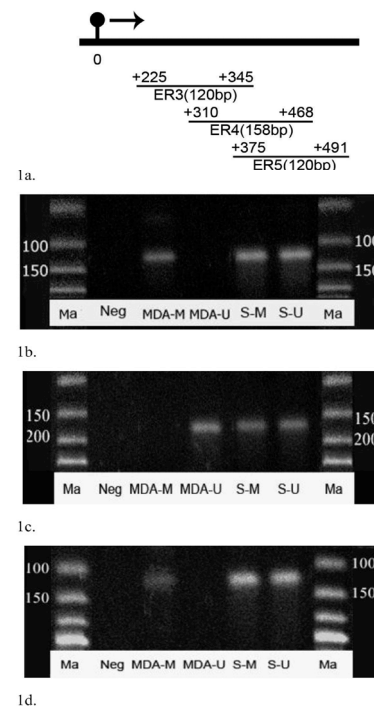
or racial primacy were selected. The nationality of all the patients was Iranian. The age of all the patients whose tumor samples were included in this study ranged from 28 to 82 years, with mean age of 47.88 years ( $\pm 10.98$ ). Patients with triple negative tumors were significantly younger than those with non-triple negative tumors ( $p=0.03$ ). 36 patients (60%) had left-side involvement. Furthermore, axillary lymph nodes were involved in 63.3% of the patients. The mean size of the tumors was  $3.43 \pm 1.75$  cm. The prevalence of grade I, II, and III tumors were 10.1, 43.3 and 46.6%, respectively. 34 (56.6%) patients were in the premenopausal age.

#### Immunohistochemistry (IHC)

Slides with  $>80\%$  malignant cells were used for immunohistochemistry. Inter- and intra- observer reproducibility in the evaluation of all the immunostaining methods was at least 95%. Specific receptors for estrogen and progesterone were detected in 31 and 40% of the tumors, respectively. Her-2 overexpression with strong circumferential staining of the entire tumor cell membranes, creating a fishnet pattern, was found in 44.6% of the tumors. Nuclear accumulation of p53 was found in 31% of the cases.

#### Methylation of ERs

ER methylation was not observed in the genomic DNA from normal breast tissues, but was found in in vitro methylated DNA and selected breast cancer cell lines (Figure 1). Methylation of ER3 was found in 25 samples (41.7%). The methylated form in premenopausal patients (1.1%) was higher than that in postmenopausal females



**Figure 1. Methylation Status of Different CpG Islands in ER-alpha Gene Was Checked Using Methylation Specific PCR.** a. Diagrammatic representation of studied ERs; b-d. Ethidium Bromide stained agarose gels showing methylated and unmethylated PCR products for ER3, ER4 and ER5, respectively. MDA-MB 468 breast cancer cell line was used as positive and no DNA as negative control

(39.1%), but the difference was not significant ( $p>0.05$ ).

The rate of ER3 methylation in the right breast tumors was significantly higher than that in the left side ( $p<0.04$ ). Premenopausal patients showed a similar pattern and even stronger difference ( $p<0.001$ ), while no significant difference was revealed in the postmenopausal ones ( $p>0.05$ ). Furthermore, no significant correlations were found between ER3 methylation status and prognostic factors, such as tumor size and grade, lymph node involvement, and IHC markers, including ER, PgR, HER-2, and p53 ( $p>0.05$ ). In addition, no significant difference in ER3 methylation status was found between triple negative and non-triple negative tumors in either pre- or postmenopausal patients ( $p>0.05$ ).

Only 7 samples (11.6%) demonstrated methylated ER4, and its frequency in postmenopausal patients (15%) was higher than premenopausal females (8.8%), but the difference was not significant. Similar to ER3, a significant difference was found in ER4 methylation status between the right and left breast tumors, and higher methylation rate was found in the right breast tumors ( $p<0.003$ ). When compared with postmenopausal females, such significant difference was found only in the premenopausal patients ( $p<0.001$ ). Interestingly, a significantly higher rate of ER4 methylation was found in p53<sup>+</sup> tumors than p53<sup>-</sup> tumors ( $p<0.04$ ).

No significant correlations were found between ER4 methylation status and prognostic factors, such as tumor size and grade, lymph node involvement, and IHC markers, including ER, PgR, and HER-2 ( $p>0.05$ ). Furthermore, no significant difference in ER4 methylation status was found between triple negative and non-triple negative tumors in pre- and postmenopausal patients ( $p>0.05$ ).

The highest methylation rate was found in ER5 (43.3%), which was significantly higher in postmenopausal females (57.7%) than premenopausal ones (32.4%) ( $p<0.032$ ). Methylation of ER5 in pre- and postmenopausal patients with bigger size or higher grade tumors was significantly higher than patients with smaller size or lower grade tumors ( $p<0.04$  and  $p<0.001$ , respectively) (Table 2). In addition, the rate of ER5 hypermethylation in pre- and postmenopausal patients with lymph node involvement was significantly higher than those with intact lymph node ( $p<0.005$  and  $p<0.004$ , respectively) (Table 2).

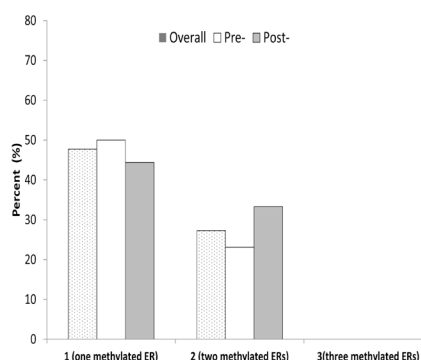
Significantly higher rate of ER5 methylation was found in patients with HER-2<sup>+</sup> tumors than those with HER-2<sup>-</sup> ( $p<0.007$ ). Significantly higher ER5 methylation was found in postmenopausal patients with ER<sup>-</sup>, PgR<sup>-</sup>, or ER<sup>-</sup>/PgR<sup>-</sup> tumors, when compared with those with non-ER<sup>-</sup>, non-PgR<sup>-</sup>, or non-ER<sup>-</sup>/PgR<sup>-</sup> ( $p<0.03$ ,  $p<0.03$ , and  $p<0.04$ , respectively). No significant difference in ER5 methylation status was found between triple negative and non-triple negative tumors in pre- or postmenopausal patients ( $p>0.05$ ). In addition, no significant correlation was found between ER5 methylation status and nuclear accumulation of p53 ( $p>0.05$ ) (Table 2).

*Methylation score*

Methylation score of the samples revealed that CpG islands of ER- $\alpha$  are methylated in 71.7% of the Iranian

**Table 2. Relationship Between ERs Methylation Status and Clinicopathological Characteristics**

Patients	Methylated			MI
	ER3	ER4	ER5	
<b>Overall:</b>				
Side of involvement	NS	P=0.003	NS	p=0.016
Lymph node involvement	NS	NS	p=0.01	NS
Tumor grade	NS	NS	p=0.001	NS
ER	NS	NS	p=0.03	NS
PgR	NS	NS	p=0.03	NS
HER2	NS	NS	p=0.007	NS
TNP	NS	NS	NS	NS
P53	NS	NS	NS	NS
<b>Pre-Menopausal:</b>				
Side of involvement	p=0.006	p=0.001	NS	p=0.006
Lymph node involvement	NS	NS	p=0.04	NS
Tumor grade	NS	NS	p=0.001	p=0.038
ER	NS	NS	NS	NS
PgR	NS	NS	NS	NS
HER2	NS	NS	p=0.002	NS
TNP	NS	NS	NS	p=0.05
P53	NS	p=0.04	NS	NS
<b>Post-Menopausal:</b>				
Side of involvement	NS	NS	NS	NS
Lymph node involvement	NS	NS	NS	p=0.024
Tumor grade	NS	NS	NS	NS
ER	NS	NS	p=0.03	NS
PgR	NS	NS	p=0.02	NS
HER2	NS	NS	NS	NS
TNP	NS	NS	NS	NS
P53	NS	NS	NS	NS



**Figure 2. Methylation Score of CpG Islands of Estrogen Alpha Gene.** Methylation score of CpG islands of estrogen receptor alpha gene in all, pre- and post-menopausal patients

women with IDCC of the breast. However, all-methylated status or Score 3 was not found in any samples (Figure 2). Significant difference in the methylation score of right and left breast was found in premenopausal patients, indicating higher methylation score of the right breast than the left breast ( $p<0.001$ ). In postmenopausal patients, significantly higher methylation score was found in tumors with higher grade ( $p<0.05$ ) and lymph node involvement ( $p<0.02$ ).

**Discussion**

It has been reported that 30% of primary breast cancers are ER- $\alpha$  negative at the time of diagnosis without considerable response to adjuvant therapy with tamoxifen. However, it has been shown that a fraction of initially ER- $\alpha$ -positive breast cancers lose ER expression during tumor



progression (Hanstein et al., 2004). Aberrant methylation of CpG islands that are located in the 5'-regulatory regions of the ER- $\alpha$  gene is a common cause of the absence of ER in a significant fraction of breast cancers (Giacinti et al. 2006). Aberrant hypermethylation of CpG islands in the gene promoters is now recognized as a common mechanism for suppressing the gene expression in cancer cells. Several genes involved in DNA repair (BRCA1 and GSTP1), cell-cycle regulation (p16INK4a and cyclin D2), cell adhesion (E-cadherin), receptor-mediated cell signaling (RAR- $\beta$  and THR- $\beta$ ), regulation of cell transcription (HOX5A), or other functions (RASSF1A, Twist, and HIN1), are most frequently methylated in breast carcinomas (Mehrotra et al., 2004).

However, the complexity of ER- $\alpha$  promoter is higher than that of other genes (Kos et al., 2001) and different ER- $\alpha$  methylation frequencies in breast cancers have been reported by different researchers.

In Iran, it has been reported that ER- $\alpha$  gene was hypermethylated in 18% of the invasive tumor samples (Rasti et al., 2009). However, they only studied a region that is equivalent to ER1 in the mapping study (Lapidus et al., 1998), and no significant correlation was reported between ER1 methylation status and clinicopathological factors, such as tumor size, grade, lymph node involvement, menopausal status, and hormone receptors (Rasti et al., 2009). Therefore, the current study was sought to elucidate the methylation status and clinicopathological importance of methylated ER- $\alpha$  in the CpG islands other than the reported ER1, in Iranian women with breast cancer. Although a relatively unimodal distribution of methylation frequency for all three histological types was reported for breast cancer (Bae et al., 2004), only IDCC, reported as the most common form of cancer in Iran (Mousavi et al., 2008), was included in the current study.

The methylation score of the samples revealed that 71.7% of the samples have at least some degree of methylation. Although our findings are not very much consistent with those of a Chinese study (Zhao et al., 2008), we found no significant difference between the methylation score of ER-positive and ER-negative tumors. On the other hand, in postmenopausal patients, we found significantly higher methylation score in tumors with higher grade and lymph node involvement. Interestingly, the methylation score of right breast cancer in premenopausal patients was significantly higher than that of left side, which has never been reported yet.

A similar study was carried out by Wei et al to investigate the methylation status of CpG islands corresponding to the ER1, 3, 4, 5 mapping scheme, and they reported that 47.4% of breast tumors with different histological types have methylated status for at least two of the ERs. They also found that methylation in tumor with higher grade is significantly higher than that in lower grade tumors. Meanwhile, a significant correlation between the hormone receptors and methylation status was also reported (Wei et al., 2008)

With regard to the methylation status of ER3, 41.7% of the samples were methylated in our study, and no significant correlation was found between ER3

methylation status and prognostic factors, such as tumor size and grade, lymph node involvement, and IHC markers, including ER, PgR, HER-2, and p53. Higher methylation rate of ER3 in the right breast of premenopausal patients was prominently significant. In another study, methylated ER3 was reported in 46% of the samples and no significant correlation between the status of methylation was found with regard to the hormone receptors and prognostic factors (Parrella et al., 2004).

We only found methylated ER4 in 11.7% of the samples, which was slightly higher in postmenopausal patients, when compared with that in premenopausal females. In other studies, methylated ER4 was reported in 24–32.6% of the tumor samples and 22% of the serum samples (Mirza et al., 2007; Zhao et al., 2008). However, no significant correlations between ER4 methylation status and prognostic factors, such as tumor size and grade, lymph node involvement, and IHC markers, including ER, PgR, and HER-2 were observed, but a significant association between ER- $\alpha$  methylation with the loss of ER- $\alpha$  protein expression was reported (Mirza et al., 2007; Zhao et al., 2008).

For the first time, we found that ER4 in tumors with nuclear accumulation of p53 are significantly methylated. Furthermore, a significant correlation was also found between ER-negative tumors and nuclear accumulation of p53, which shed light on the importance of ER methylation on poor prognosis of breast tumors.

In case of ER5, we found that it is methylated more frequently than ER3 and 4. The highest methylated status for ER5 was found in postmenopausal patients and was significantly higher than that of premenopausal females, and showed an age-dependent pattern. Significantly higher methylation of ER5 in tumors with bigger size or higher grade, with lymph node involvement, ER, PR, and ER/PR negativity makes it a good candidate as a prognostic factor in patients with ductal cell carcinoma of the breast.

Interestingly, we found that ER5 in patients with HER-2+ tumors is significantly methylated. Although HER-2 positivity confers an extra therapeutic chance for patients, it is a growth factor receptor and tumors with higher level of receptors tend to grow faster. Therefore, the observed association between methylation of ER and higher grade of tumors is consistent with the association between ER methylation status and HER-2 positivity, and shows that prognosis of tumors with methylated ERs are poorer than those with unmethylated ERs.

Another question that we addressed in the current study was the possibility that methylation patterns of different ERs could be used as a prognostic factor in triple negative breast cancers that lack expression of ER, PR, and HER-2 and represent about 15% of all breast cancers (Viale & Bottiglieri 2009; Seal & Chia, 2010) with biologically aggressive phenotypes and limited treatment options (Conte & Guarneri, 2009). To approach this issue, we compared the methylation status of ERs and methylation score of triple negative tumors with non-triple negatives. To our knowledge, this is the first report that had paid specific attention to the impact of triple negative phenotype on the methylation status of ER- $\alpha$ .

Our findings showed that the methylation status of

different CpG islands in the promoter region of ER- $\alpha$  in tumors with triple negative phenotype was not significantly different from those with non-triple negative phenotypes. Significant impact of triple negative phenotype was found neither in the pre- nor postmenopausal patients. Reverse association between the methylation status of ERs and hormone receptors and HER-2 overexpression could be considered as an acceptable explanation. No other study on the impact of triple negative phenotype on the methylation status of ER was found, and hence, we were unable to compare our results with others; however, a significant decrease in the hormone receptors along with a significant increase in HER-2 expression in IDCC of the breast has been reported earlier (Liu et al., 2009).

In the current study, the mean age of all the patients was  $47.88 \pm 10.98$  years, which is consistent with the reported results (Vahdaninia & Montazeri, 2004; Mousavi et al., 2006), and proves that the onset of breast cancer in Iranian women is a decade earlier, when compared with those in Western countries (Harirchi et al., 2000; Mousavi et al., 2007b; Kollahdoozan et al., 2010). However, a significant correlation and an increasing trend of methylation were found for ER5 in elder patients than the younger ones.

Although a significant correlation was found between ER5 methylation and in spite of the lack of ER protein in the current study, it is known that promoter methylation is not the only mechanism required for gene silencing. Furthermore, it should be considered that the genomic organization of the ER- $\alpha$  gene is much more complex than expected. Consequently, interplay between the known promoters and their transcriptional regulators is suggested as an explanation for the observed complex and confusing results of different studies (Parrella et al., 2004).

Different and somehow arbitrarily designed scoring systems and cutoff points for IHC assessment of hormone receptors are the important sources of discrepancy in the published reports. A meta-analysis study is suggested to refine the current ambiguity.

A survey of the literature shows that the reported frequency of CpG island methylation in the promoter region of ER- $\alpha$  in Chinese (Zhao et al., 2008), Turkish (Buyru et al. 2009), North American and Korean (Lee et al., 2008), American (Wei et al., 2008), and Indian (Mirza et al., 2007) women with breast cancer is different, and a diverse impact on the prognosis of cancer has also been reported in different countries. The frequency of methylation in different ER in the current study is not exactly similar to the previously reported studies, but considerable similarities of methylation frequency of each ER, i.e. ER3, ER4, and ER5, has been found between the current study and some earlier studies. For example, our findings for ER4 methylation frequency are different from those of a Chinese study (Zhao et al., 2008), and similar to those of Indians (Mirza et al., 2007). A unique ethnic profile for aberrant methylation of CpG islands in ER- $\alpha$  might be a possible reason for the observed difference in various countries.

At the same time, method-related deviations in the results could be considered as another source of difference in the reported methylation frequencies. MSP, as a sensitive method of methylation detection in CpG

islands (Herman et al. 1996), is the most frequently used method in different investigations, such as the current study, but old techniques, such as blotting-based methods or recently used methods such as real-time PCR or methyl light based techniques have also been applied in several studies. Therefore, a meta-analysis study is suggested to clarify the existent confusing picture.

In conclusion, results of the current study, as the first methylation mapping of CpG islands in ER- $\alpha$  in Iranian females with IDCC of the breast, revealed that silencing of this gene by promoter hypermethylation is a frequent event, but its frequency in each of the different CpG islands is different. Results revealed a proof of independence of CpG island methylation in right and left breast tumors with preferential patterns. Furthermore, the most accepted clinicopathological or prognostic characteristics were included in the study and a diverse impact of aberrant hypermethylation of different CpG islands on the prognosis of breast cancer was found. We found that ER5 is not only the most frequently methylated region, but also has a significant impact on the prognostic factors of breast cancer, such as tumor size and grade, lymph node involvement, hormone receptors, and HER-2 overexpression. Therefore, methylated ER5 could serve as a marker of poor prognosis.

Significant difference in ER methylation status was not found between triple negative and non-triple negative tumors. Further investigations with larger sample size are suggested to re-evaluate the detected effect of the side of involvement on the methylation status of ER.

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