RESEARCH ARTICLE

Association of Poor Prognosis Subtypes of Breast Cancer with Estrogen Receptor Alpha Methylation in Iranian Women

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Abstract

 Breast cancer is a prevalent heterogeneous malignant disease. Gene expression profiling by DNA microarray can classify breast tumors into five different molecular subtypes: luminal A, luminal B, HER-2, basal and normal-like which have differing prognosis. Recently it has been shown that immunohistochemistry (IHC) markers including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2), can divide tumors to main subtypes: luminal A (ER+; PR+/-; HER-2-), luminal B (ER+;PR+/-; HER-2+), basal-like (ER-;PR-;HER2-) and Her2+ (ER-; PR-; HER-2+). Some subtypes such as basal-like subtype have been characterized by poor prognosis and reduced overall survival. Due to the importance of the ER signaling pathway in mammary cell proliferation; it appears that epigenetic changes in the ERα gene as a central component of this pathway, may contribute to prognostic prediction. Thus this study aimed to clarify the correlation of different IHC-based subtypes of breast tumors with ERα methylation in Iranian breast cancer patients. For this purpose one hundred fresh breast tumors obtained by surgical resection underwent DNA extraction for assessedment of their ER methylation status by methylation specific PCR (MSP). These tumors were classified into main subtypes according to IHC markers and data were collected on pathological features of the patients. ERα methylation was found in 25 of 28 (89.3%) basal tumors, 21 of 24 (87.5%) Her2+ tumors, 18 of 34 (52.9%) luminal A tumors and 7 of 14 (50%) luminal B tumors. A strong correlation was found between ERα methylation and poor prognosis tumor subtypes (basal and Her2+) in patients (P<0.001). Our findings show that ERα methylation is correlated with poor prognosis subtypes of breast tumors in Iranian patients and may play an important role in pathogenesis of the more aggressive breast tumors.

Keywords: Estrogen receptor alpha - promoter methylation - breast cancer subtypes - Iranian patients

Introduction

 The prevalence rate of breast cancer in Iranian population for women over 30 is 120 per 100,000 (Kolahdoozan et al., 2010). Age of onset of breast cancer in Iran seems to be about 10 years earlier than western countries (Harirchi et al., 2004; Mousavi et al., 2007; Mousavi et al., 2009). However until now the reason has not been fully explored and this different feature may reiterate the importance of investigation in epigenetic and genetic markers in Iranian breast cancer patients. This cancer as a heterogeneous malignant disease composed of different subtypes with diverse natural history, response to treatment and clinical outcome. Gene expression profiling has identified several distinct breast subtypes such as luminal A and B, Her-2, basal-like and normal breast like (Perou et al., 2000). Luminal A and B subtypes are estrogen receptor positive tumors, while the overall survival of patients with luminal A tumors is significantly

greater than patients with luminal B cancers. Basal-like (includes triple negative tumors) and Her2+ breast cancers are associated with overall poor prognosis (Sandhu et al., 2010). On the other hand, while molecular sub typing of breast tumors provides useful prognostic and predictive information, it is not yet widely available and cost effective. Classification of breast tumors by immunohistochemistry (IHC) analysis of estrogen and progesterone receptors and Her2 amplification could reproduce the results of classification based on gene expression microarray panel (Vallejos et al., 2010). Although IHC markers are less sensitive, they can be useful and accessible surrogate markers of gene expression analysis. Actually, based on many efforts to categorize breast tumors with surrogate IHC markers (Carey et al., 2006; Vallejos et al., 2010; Voduc et al., 2010), it has been shown that molecular subtypes approximated by IHC markers can predict the prognosis of breast cancer patients (Chen et al., 2010).

 Estrogen and its receptor have an important role in the

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pathogenesis of breast cancer. Indeed ERα is the central part of an important signaling pathway in mammary cells (Hayashi et al., 2009). At least 100 proteins are known to interact with ERα (Badve et al., 2009). It encodes by a 140 kb gene on the 6q25.1 chromosome (Kos et al., 2001). It is known that the initiation of malignancy in breast as well as its transition towards distinct breast cancer subtypes with different prognosis is triggered by the accumulation of many genetic and epigenetic abnormalities (Veeck et al., 2010). Epigenetic changes, especially CpG island methylation in tumor suppressor genes are one of these potential mechanisms. ERα gene as an important gene in pathogenesis of breast cancer is one of the targets of aberrant epigenetic changes in breast tumors (Ramezani et al., 2012). As it has never been investigated in different IHC-based subtypes of breast tumors in Iranian patients, we aimed to evaluate the role of ERα promoter methylation in four main subtypes of breast cancer in Iranian women who affected one decade earlier than their western counterparts.

Materials and Methods

Patients

The study population consisted of 100 patients with sporadic primary breast cancer. Tumor samples were provided by the Iranian National Tumor Bank. Written informed consent was given by all the patients enrolled in the study. This study was approved by the local ethical committee at Tarbiat Modares University. None of patients underwent radiotherapy, chemotherapy or adjuvant treatment before surgery. Tumor samples were obtained by surgical resection and were transferred to surgical pathology ward of cancer institute. An expert pathologist performed rapid macro dissection of samples and transferred malignant tissues to liquid nitrogen tank immediately.

Clinico pathological data collection

The collected data included patient's age, menopausal status and clinic pathological features (tumor size, histological type, grade, lymph node involvement, stage and immunohistochemistry panel consisted of ER, PR, Her2 and p53). All analyses were performed in a single referral laboratory using the same method to exclude the problem of inter laboratory variations. Tumor size was determined from the largest diameter in the gross sample (T1≤2 cm, T2=2.1-5 cm, T3>5 cm). Histological type and grade were determined by a single pathologist and rechecked and approved by another expert. ER and PR were considered negative when nuclear staining of malignant cells was below 1%. P53 was considered positive when nuclear staining was positive in more than 5% of malignant cells. Her2 over expression was considered positive when continuous and strong membrane staining was observed in the more than 30% of tumor cells.

Categorization of breast tumors subtypes by immunohistochemistry markers

Breast tumors were classified based on immunohistochemical surrogate markers(ER, PR and HER2) to determine the molecular subtypes (Sandhu et al., 2010). These tumors were classified to luminal A (ER and/or PR+, Her2-), luminal B subtype (ER and/or PR+, Her2+), Her2+ subtype (ER-, PR-, Her2+) and basal subtype (ER-, PR-, Her2-).

DNA extraction and bisulphite modification

Genomic DNA was extracted from frozen breast tumors by High pure PCR template preparation kit (Roche, Germany) according to manufacturer's instructions. The quality and the quantity of extracted DNA were verified by spectrophotometer (Nano drop 2000, Thermo scientific, USA). One μg of genomic DNA of each sample was used for Sodium bisulphite modification as previously described (Herman et al., 1996).

Methylation Specific PCR (MSP)

ER3 region in 5´UTR of proximal promoter of ERα gene was selected based on previous studies (Lapidus et al., 1998; Sorlie et al., 2001; Zhao et al., 2009). Primer pairs and PCR condition were previously described (Lapidus et al., 1998). MSP reaction was optimized in our lab with 1xPCR buffer, 6.7 mM MgCl2, and 1.25 mM dNTP per 25 μl reaction volumes. 2.5 units Taq DNA polymerase (Cinaclone, Iran) was added to each reaction after initial denaturation (manual hot start). Positive control DNA for MSP with methylated primers was extracted from MDA-MB-231 cell line which had been shown in previous works to be totally methylated in 5´UTR of ERα (Lapidus et al., 1998). Negative control DNA for MSP with methylated primers was extracted from MCF-7 cell line. Blank or no DNA reaction was used as negative control to control template contaminations. Untreated DNA was used as the control of specificity of reactions. Amplification products were electrophoresed on a 1XTBE/2% agarose gel stained with ethidium bromide and visualized under UV light. Tumors were classified as methylated if they had amplification for methylated primers.

Statistical analysis

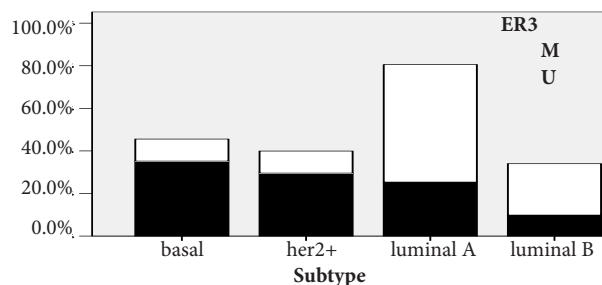Association between ERα methylation and different molecular subtypes was analyzed by Pearson Chi-Square and Fisher's exact test using SPSS v.13. P value less than 0.05 was considered as significant and confidence intervals quoted were at the 95% level.

Results

Breast tumors were classified based on IHC surrogated markers to 34 luminal A, 14 luminal B, 24 Her2+ and 28 basal subtypes. Then the methylation status of ERα promoter CpG island in ER3 region was determined in these subtypes using the MSP method. ERα methylation was detected in 52.9% (18 tumors out of 34) of luminal A subtype, 50% (7 tumors out of 14) of luminal B subtype, 87.5% (21 tumors out of 24) of Her2+ subtype and 89.3% (25 tumors out of 28) of basal subtype (Figure 1). We could show a significant correlation between ERα methylation and poor prognosis, non luminal subtypes (basal and

Table 1. Association between Different Subtypes and Clinicopathologic Features in Studied Population (NS means not significant)

Features		Luminal subtype A (n=34)	Luminal subtype B (n=14)	Her2+ subtype (n=24)	Basal-like subtype (n=28)	P value
Age at diagnosis(year)	≤50	19	9	17	11	NS
	>50	15	5	7	17	
Menopause status	Pre -menopause	16	9	14	11	NS
	Post -menopause	18	5	10	17	
Tumor size(cm)	≤2.0 cm (n=16)	6	3	4	3	NS
	2.1-5 cm (n=57)	22	7	11	17	
	>5 cm (n=27)	6	4	9	8	
Tumor grade	I	14	4	7	1	0.03
	II	13	7	8	13	
	III	7	2	9	12	
	X	0	1	0	2	
P53 status	Negative	18	6	8	16	NS
	Positive	16	8	15	12	
Stage	1	4	2	2	2	NS
	2	16	2	9	19	
	3	13	9	11	7	
	4	1	0	2	0	

Figure 1. Differences in ER3 Methylation in Various Breast Tumors Subtypes (Black Area=Methylated or M & White Area = Unmethylated or U).

Her2+) in our studied population (p=0.001). There was no association between ERα methylation and patients' age, menopausal status, tumor size, tumor grade and stage. Association between clinicopathological features of studied tumors with different subtypes have evaluated in Table 1. The correlations of patients' age and menopausal status with tumors subtypes were not significant in the studied population. Meanwhile there was no statistical significant correlation between tumor size and different subtypes; larger tumors (more than 5 cm) have been shown in 17% of luminal A, 28% of luminal B, 28% of basal-like and 37% of Her2+ subtypes. It seems that tumors with Her2+ subtype tended to present with larger tumor size. Also there was a significant association between tumors grade and basal-like subtypes (p=0.03). The association between p53 IHC status in tumors and subtypes was not significant but p53 positive status (p53 nuclear accumulation) was more prevalent in Her2+ subtype tumors (65.2%) than others subtypes.

Discussion

Classification of breast tumors based main molecular subtypes was a promising tool for more precise prediction of clinical outcome and overall survival (Sorlie et al., 2001; Ihemelandu et al., 2007). Molecular sub typing based on gene expression profiling is not cost effective and needs special requirements which are not feasible for all diagnostic laboratories. Previous studies showed that using of routine IHC markers (ER, PR, HER-2) as surrogate markers, maybe useful to classifying breast tumors to molecular subtypes (Carey et al., 2006; Vallejos et al., 2010; Voduc et al., 2010). As it has been shown that ER CpG island methylation in Iranian breast cancer patients is a marker of poor prognosis (Ramezani et al., 2012), we tried to investigate the correlation of this epigenetic marker to different tumors subtypes in Iranian studied population. The age of onset in Iranian breast cancer patients is one decade earlier than western patients (Mousavi et al., 2009) and according to our knowledge it is the first report of correlation of an epigenetic marker with breast cancer subtypes in this population. We found in this research that ER promoter methylation is strongly correlated to poor prognosis (Her2+ and basal-like) subtypes of breast cancer in Iranian patients. Global methylation deviation is a marker of bad prognosis and ER methylation status is considered as an independent marker of malignant biology (Killian et al., 2011). This difference in DNA methylation in promoter regions and the correlation to subtypes has been observed in other genes (Hill et al., 2011). Recently, researchers have shown that methylation levels in four genes are dissimilar in Korean patients with breast cancer when comparing HER2 and luminal subtypes (Lee et al., 2010). They concluded that gene methylation in breast cancer can potentially serve as epigenetic biomarkers and may contribute further to current breast cancer classification. However, in contrast to our results, they failed to find a correlation between the ERα promoter methylation and the molecular subtypes of breast cancer. Ethnic differences in the studied populations may explain this difference.

Also, there are some evidences to believe that ER promoter methylation can be different in molecular subtypes of breast cancer. In a recent study it has shown that epigenetic differences especially in ERα exist between expression subtypes of breast cancer (Ronneberg et al.,

2011). Our results confirm that the methylation of ERα promoter changes in molecular subtypes of cancer and emphasize the role of ERα methylation as a marker of prognosis in breast cancer. It has been shown that ER negative breast tumors are more prevalent in younger patients and in Iranian breast cancer patients. In previous researches it has been shown in Iranian patients that38-43% of tumors are estrogen receptor negative (Fallahazad et al., 2004; Vahdaninia et al., 2004; Onitilo et al., 2009). Even if these researches suffer from limited number of included cases, they are in concordance to other works showing the influence of ethnicity on breast tumor characteristics (Hines et al., 2011). It has been shown previously that ER signaling pathway plays an important role in the pathogenesis of breast cancer and ERα protein expression in breast tumors has been widely accepted as a predictor for response to endocrine therapy and a prognostic marker in breast tumors (Travis et al., 2003; Dunnwald et al., 2007; Polyak et al., 2007).

In our study, there was no association between patients' age and ERα methylation, thus this epigenetic phenomenon is not an age-related event in Iranian breast cancer patients. The relationship between age and ERα methylation in different studies were controversial. In one study on 193 Australian patients (Li et al., 2006), it was associated with younger patient's age. In other studies there was no correlation between ERα methylation and age in breast tumors (Iwase et al., 1999; Parrella et al., 2004; Mirza et al., 2007; Wei et al., 2008; Zhao et al., 2009). In spite of observations which have shown that ERα methylation can be an epigenetic molecular clock in some tissues such as colonic tumors and cardiovascular epithelium (Issa etal., 1994; Post et al., 1999), it seems that ERα methylation in breast tumors is age-independent. Also we have shown that ERα methylation is independent from menopausal status, tumor size, grade and stage in concordance to other researchers (Li et al., 2006; Mirza et al., 2007; Wei et al., 2008; Zhao et al., 2009).

Presence of ERα methylation in a sizable fraction of luminal A and B subtypes which are ER positive, maybe due to cellular heterogeneity in breast tumors. ER status in tumors is a continuous variable which has been dichotomized in dividing tumors to ER negative and ER positive. Such a classification may cause partial loss of information (Gown, 2008). Presence of ERα methylation in luminal A and B subtypes is a manifestation of this cellular heterogeneity and may contribute to endocrine therapy resistance or recurrence. More investigations are needed to clarify the contribution of ERα methylation in endocrine therapy resistance and recurrence in luminal subtypes breast tumors.

In this study in spite of no statistical significant association between tumor size and subtypes, we have seen that Her2+ tumors tend to have larger (more than 5 cm) size in our studied population. This phenomenon also has been observed in Thai breast cancer patients (Chuthapisith et al., 2012). Also we have shown that the most of the basal-like tumors are high grade in concordance with other studies (Chuthapisith et al., 2012). Nuclear accumulation of p53 (positive status of p53 in

IHC) is another marker for poor prognosis in cancer and many mutations in p53 gene can cause accumulation of this protein in nuclease. Although the correlation of p53 positive status with different subtypes didn't reach to statistical significant level; it was more prevalent in Her2+ subtype. This finding needs more investigation in a larger population of breast cancer patients.

However, in this study we had limitations about classification of tumors to different subtypes based on their IHC panel. Although IHC-based categorization of breast tumors is more practical and cost effective; it may not be very accurate like classification based on gene expression profiling by microarray. Use of more IHC markers such as Ki-67 and proliferating cell nuclear antigen in sub typing of breast tumors is suggested for more precise classification.

In conclusion our study showed that methylation of ERα is a prevalent epigenetic phenomenon in Iranian breast cancer patients with poor prognosis tumors such as Her2+ and basal-like subtypes. In addition, presence of ER methylation in a sizable fraction of luminal A and B subtypes of breast tumors shows their heterogeneous nature. The relationship between this epigenetic event and resistance to hormone therapy or recurrence in luminal subtypes needs more investigation in the future.

Acknowledgements

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