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Estrogen Receptor-alpha mRNA in Primary Breast Cancer: Relationship to Estrogen and Progesterone Receptor Proteins and Other Prognostic Factors

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SUMMARY **The estrogen receptor (ER)-alpha protein and *ER* mRNA were measured in 314 primary breast cancer patients by enzyme immunoassay (EIA) and reverse-transcription polymerase chain reaction (RT-PCR) assay, respectively. The positivity of ER protein was 53% while of *ER* mRNA was 37.6%. A significant positive association between ER phenotype and *ER* mRNA was observed ($r = 0.40$, $p < 0.0001$) with a positive-negative agreement between them of 71.8%. The percentage of ER-negative, progesterone receptor (PR)-positive breast tumors was 1.9% by EIA and 7% by RT-PCR assay. This may indicate a difference in *ER* variants in these studied patients. The ER protein and *ER* mRNA status were inversely related to tumor size and p53 positivity. Also, ER protein was frequently positive in patients with a higher number of lymph node invasions, well to moderate nuclear differentiated tumor cells and negative c-erbB-2 status. The difference of the ER or *ER* mRNA status regarding ages, menopausal status, tumor stages and histological types was not shown. In the present study, *ER* mRNA did not demonstrate a closer relationship to prognostic indicators of breast cancer than ER protein. Before including the *ER* mRNA assessment in routine investigations of breast cancer, its relationship to prognostic factors and survival outcome should be further assessed with a higher number of patients and a longer follow-up time.**

Estrogen receptor (ER) is a 65 kDa protein with its amino acid sequence alignment grouped in six domains, A to F. The human ER is encoded by a large gene split into 8 exons.[1] The DNA binding domain which allows the ER protein to coordinate with the estrogen response elements is coded by exons 2 and 3.

It has been demonstrated that estrogen action is mediated not only through a classic ER, named ER-α but also through a second ER (ER-β) that has a structure and function similar to ER-α.[2] The DNA binding domain of the two ER has 95% amino acid identity and both ER may form functional heterodimers.[3-4]

The presence of ER in breast tumor cells is considered a good prognosis and the patients who express ER in their tumor have an overall longer survival and lower risk of tumor recurrence.[5] ER is important not only for the prognostic value but also for the outcome of endocrine therapy in breast carcinoma.

The limited amount of tumor tissue available from biopsy or surgery often restricted ER protein quantitation by the previous methods including radioligand assay (RLA), enzyme-immunoassay (EIA) or immuno-histochemical assay (IHC). The EIA and IHC detect the physical presence of ER when epitopes recognized by the antibodies remain intact; these receptors can therefore be functionally inactive.

Recently, a polymerase chain reaction (PCR)-based method for the detection and relative quantitation of *ER* mRNA in breast tumor has been developed.[6-9] *ER* mRNA exists as wild-type (full-length) and alternatively spliced

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variants in breast tumor tissue.[9-10] Some *ER* variants can activate transcription in the absence of hormone. This may be responsible for an ER-negative/progesterone receptor (PR)-positive phenotype of breast tumors detected by classical receptor assays.[11] Alternatively, another *ER* variant in ER-positive phenotype of breast tumor is unable to function as a transcriptional inducer of PR protein expression.[11] Measurement of ER protein, thus, may not absolutely be an indicator of their function. The PCR technique for examining expression of *ER* mRNA has been suggested as a good screening method for the detection of constitutively active ER.[6-9]

Most clinical data concerning the prognostic significance of ER in breast cancer were evaluated in terms of ER-α. The IHC assay and the EIA for ER have been considered to measure ER-α but not ER-β.[12-13] The radioligand assay measured both subtypes of ER but ER-α in the majority of cases.[13-14]

We have developed a reverse transcription (RT)-PCR method to detect *ER*-α mRNA.[15] The relationship between *ER* mRNA and ER protein as well as other clinico-pathological parameters of breast tumor have been evaluated. To our knowledge, this is the first study to evaluate the clinical usefulness of *ER* mRNA measurement in breast cancer of Thai women.

METERIALS AND METHODS

Patients and tumor samples

Three hundred and fourteen primary breast tumor tissues were obtained from patients aged 26-83 years within 30 minutes after breast operation for measurement of ER protein by EIA (Abbott laboratories) and PR protein by RLA.[16] The ER and PR status were recorded as negative when values below 15 and 10 fmol/mg protein, respectively, were obtained.

Other clinico-pathological data collected included patient ages, menopausal status, surgical procedure, tumor size-lymph node-metastasis (TNM) clinical and pathological classifications for tumor stages and nuclear differentiation grade.

RNA isolation and RT-PCR

Total RNA was isolated from approximately 50 mg of tumor tissue and about 0.2 to 5 μg/ml amount of RNA was obtained from each sample. Reverse transcription of RNA to DNA was performed in the same tube for DNA amplification. The sense *ER* primer was 5'-ACTCGCTACTGTGCAGTGTGC-AATG-3' corresponded to *ER* cDNA sequence 776-800.[17] The antisense primer of the *ER* cDNA sequence 1014-990 was 5'- CCTCTTCGGTC-TTTTCGTATCCCAC-3'. The cDNA sequences of two *ER* primers were within exon 2 and exon 3 that encoded the DNA binding domain of *ER*-α. The sense β_2-microglobulin (β_2M) primer corresponding to cDNA 97-116 and the antisense β_2M corresponding to cDNA 242-261 were used as internal control for RNA extraction and relative quantitation of *ER* band-intensity.[18] The PCR product was run on agarose gel and stained with ethidium bromide. The ratio of *ER* over β_2M band-intensity detected by video Gel Documentation System (Gel Doc 1000, Bio-Rad) was calculated. The procedure for RNA isolation and RT-PCR used was modified from Chevillard's group,[7] and the details of the procedure were published elsewhere.[15]

Statistical analysis

The *ER* mRNA level and other studied parameters were expressed as mean ± SEM. Comparison of values among different groups was performed by non-parametric methods. The correlation between corresponding value of log *ER* mRNA level and log ER protein concentration was tested by Spearman's rank correlation. The significance for group comparison was calculated by Chi-square method. Two-side $p < 0.05$ was considered as statistically significant. Statview PC 4.5 was used for statistical analyses.

RESULTS

Values of ER measured by EIA and RT-PCR methods are shown in Table 1. The median value of *ER* mRNA was zero. A significant correlation between log *ER* mRNA levels and log ER protein concentrations was detected ($r = 0.40$, $p < 0.001$) (Fig. 1). The cut-off value 15 fmol/mg by EIA was equivalent to a 0.6 ER/β_2M ratio in the RT-PCR assay.

The *ER* mRNA/β_2M ratio was recorded as positive whenever the bands could be read (above zero value). This cut-off gave a better sensitivity in comparison to the 0.6 ratio value. The comparison of *ER* mRNA against the ER protein status is shown in Table 2. The sensitivity (true positivity rate) by RT-PCR assay was 59.5% but the specificity (true negativity rate) was 90.6%. The agreement between the two methods was 71.8%.

The frequency of *ER* mRNA positivity was counted at different ER protein levels (Table 3). An ER protein concentration of less than

Table 1 ER protein and *ER* mRNA levels in 314 breast cancer tissues

	ER protein (fmol/mg protein)	*ER* mRNA (*ER*/β_2*M* ratio)
Mean	47.74	1.09
SEM	3.97	0.18
Mode	0	0
Median	18.51	0
Minimum	0	0
Maximum	304.44	37.91

Table 2 Validation of RT-PCR assay for *ER* mRNA in comparison to ER protein status by EIA.

		% (N)
ER-protein/*ER* mRNA	+ / +	31.7 (98)
	+ / -	21.6 (69)
	- / +	6.6 (21)
	- / -	40.1 (126)
RT-PCR	Sensitivity	59.5
	Specificity	90.6
	Positive predictive value	88.2
	Negative predictive value	65.4
	Positive/negative agreement	71.8

Table 3 Percentage of *ER* mRNA positivity classified by ER levels

	% *ER* mRNA positivity
ER- protein level (fmol/mg protein)	
0 - 9.99	14.6
10 - 14.99	16.7
15 - 19.99	46.2
20 - 29.99	68.0
30 - 49.99	63.3
≥ 50	62.7

$p < 0.0001$

15 fmol/mg yielded a significant lower percentage of positivity compared to the level above 15 fmol/mg. The positivity of ER protein was 53.2% and 37.9% for *ER* mRNA (Table 4). The agreement between ER and PR status which should be an indicator for functioned ER was slightly better for *ER* mRNA (63.4%) than for ER protein (58%).

The *ER* mRNA levels were grouped into 3 classes of *ER*/β_2*M* ratio: low (0-0.14), medium (0.15-1.99), and high (>2.0) ratios (Table 5). Of 147 ER-negative tumors, 85.7% had low-level *ER* mRNA while 58.7% among 167 ER-positive tumors had medium- to high-level *ER* mRNA. Within the low *ER* mRNA group, 64.6% expressed an ER-negative phenotype whereas 93.2% of the high *ER* mRNA group were characterized as ER-positive. PR as well as ER status differed between low- and medium-level *ER* mRNA ($p = 0.0180$ and $p < 0.001$, respectively). ER but not PR status was also different between medium- and high-level *ER* mRNA ($p = 0.0132$).

The discrepancy of ER status between the two measuring methods is shown in 90 tissues (Table 6). In relation to PR, ER protein disagreed with PR in 53 out of 90 samples (58.9%) whereas *ER* mRNA disagreed with PR in 37 samples (41.1%). PR protein positivity agreed better with ER protein than *ER* mRNA (13.4 % *vs* 8.3 %) but PR protein negativity was more agreeable to *ER* mRNA than ER protein (55 % *vs* 45 %).

An association between the ER results from each method with clinical data was examined as shown in Table 7. Both ER protein and *ER* mRNA status significantly differed between small and large

Table 4 PR and ER status by each measuring method (N = 314)

		ER (EIA)*		*ER* mRNA (RT-PCR)**	
	N	% Positive (N)	% Negative (N)	% Positive (N)	% Negative (N)
PR-RLA					
Positive	48	87.5 (42)	12.5 (6)	54.2 (26)	45.8 (22)
Negative	266	47.0 (125)	53.0 (141)	35.0 (93)	65.0 (173)
Total	314	53.2 (167)	46.8 (147)	37.9 (119)	62.1 (195)
PR ER agreement		58.3 (183)		63.4 (199)	

*$p < 0.0001$; **$p < 0.01$

Table 5 Association between different *ER* mRNA levels and ER PR protein status as determined by conventional assays

	No. of patients	*ER* mRNA (%)		
		Low (N)	Medium (N)	High (N)
ER-negative	147	85.7 (126)	12.2 (18)	2.1 (3)
ER - PR -	141	85.8 (121)	12.1 (17)	2.1 (3)
ER - PR +	6	83.3 (5)	16.7 (1)	0 (0)
ER-positive	167	41.3 (69)	34.1 (57)	24.6 (41)
ER + PR -	125	41.6 (52)	32.8 (41)	25.6 (32)
ER + PR +	42	40.5 (17)	38.1 (16)	21.4 (9)
Total	314	62.1 (195)	23.9 (75)	14.0 (44)

$p < 0.0001$

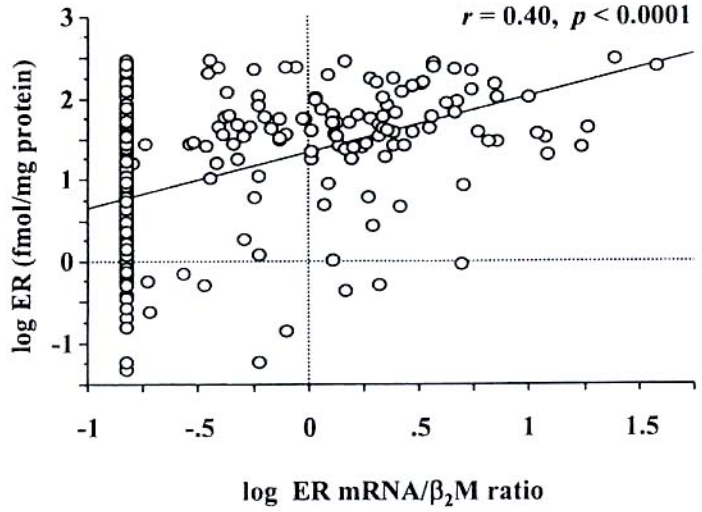
Table 6 Distribution of ER and PR status among 314 breast cancer samples

	ER-EIA	*ER* mRNA	PR-RLA	% (N)
ER Agreement (N = 224)	+	+	+	11.2 (25)
	+	+	-	32.6 (73)
	-	-	-	54.0 (121)
	-	-	+	2.2 (5)
ER Disagreement (N = 90)	+	-	-	57.8 (52)
	+	-	+	18.9 (17)
	-	+	+	1.1 (1)
	-	+	-	22.2 (20)

Table 7 Relationship between ER protein (EIA) or *ER* mRNA (RT-PCR) to some clinico-pathological and biological parameters of breast cancer

Characteristics	N	% ER protein positive	% *ER* mRNA positive
Age (years)			
< 50	161	54.7	38.5
≥ 50	154	52.6	38.4
Menopausal status			
Pre menopause	131	58.8	45.8
Post menopause	103	55.3	40.0
Tumor diameter			
< 20 mm	47	74.5[2]	61.7[2]
≥ 20 mm	176	53.4	40.5
Nodal status			
0	86	46.5[2]	41.0
1 - 3	61	63.9	41.0
3 - 10	44	75.0	50.0
> 10	36	50.0	44.4
Pathological stage			
1	19	68.4	68.4
2	174	56.3	42.4
3	27	66.7	48.1
4	1	100.0	0
Nuclear Differentiation			
Well to moderate	90	64.4[3]	45.6
Poor	73	47.9	40.3
Histology			
Invasive ductal	214	56.1	42.2
Invasive lobular	3	100.0	66.7
Other types	17	58.8	60.0
C-erbB-2 status			
Negative	116	63.8[1]	43.0
Positive	34	32.4	29.4
p53 status			
Negative	113	63.7[1]	46.9[1]
Positive	35	31.4	20.0

[1] $p < 0.001$; [2] $p < 0.01$; [3] $p < 0.05$



Regression equation : log Y = 1.337 + 0.683* log X

Fig .1 Correlation of *ER* mRNA and ER protein expression in 314 breast tumors.

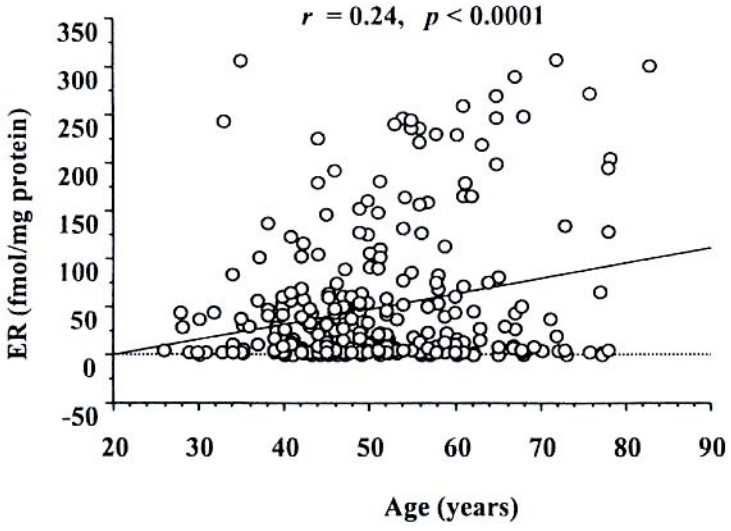


Fig. 2 Positive relationship between ER protein concentrations and age of breast cancer patients.

tumors, as well as between positive and negative p53 status. However, ER protein but not *ER* mRNA showed a significantly different levels between the number of lymph node invasions, nuclear differentiation grades as well as c-erbB-2 status. Neither ER protein nor *ER* mRNA status had a significant relationship to age, menopausal status, histological types and pathological stages in this patients' group.

DISCUSSION

An association between *ER* mRNA expression and ER phenotype shown in this study is similar to previous reports using the methods of EIA,[7-8] IHC assay,[19] or RLA.[20] Percentage positivity of ER protein by EIA (53%) obtained from Thai breast cancer patients was at the lower range of 50-80% reported among different ethnic groups.[21] The proportion of ER-positive breast cancers is higher among Caucasian than among Asian patients.[22] Nomura's group reported 56% ER-positive breast cancers in 3,089 Japanese patients.[23] In addition, the ER-α concentration in normal breast tissue of Asian women was reported to be less than in Caucasian women[24] and the authors hypothesized that a higher expression of ER in normal breast tissue increases the risk of breast cancer.

Positive *ER* mRNA was reported in 82% of breast tumors by Carmaci's group.[8] In another study, 78.4% positivity of *ER* mRNA was found in well or moderately differentiated tumors and 46.5% in poorly differentiated tumors.[20] We report 37.6% *ER* mRNA-positive breast cancer among the whole group of patients, 45.6% in well to moderately differentiated tumors and 40.3% in poorly

differentiated tumors. The ethnic differences and possibly a lower sensitivity of the RT-PCR assay used may explain the lower percentage positivity of *ER* mRNA in Thai women with breast cancer in comparison to white women.

ER mRNA correlated well with ER protein in this report. The concordance rate between the two was 71.8%. At a concentration below 15 fmol/mg of ER protein, a lower percentage positivity of *ER* mRNA was obtained (Table 3). The ER protein positivity was found more in medium- to high-level *ER* mRNA in comparison to low-level *ER* mRNA concentrations (Table 5). This disagreement which was found more at low ER protein levels may be caused by a less sensitive RT-PCR assay. Alternatively, variants of *ER*-α that could not be detected by the primers used in this assay might be another cause for the differences between the *ER* mRNA and ER phenotypes.

The presence of PR in breast tumors suggests that ER are not only present but also functional. The absence of PR is associated with a markedly reduced response to endocrine therapy.[25] In the present study, PR seems to have a better agreement with *ER* mRNA than ER protein status. However, further investigations concerning an association between *PR* mRNA and PR protein as well as between *ER* mRNA and *PR* mRNA are necessary.

About 10% of ER-negative breast cancers measured by EIA respond to hormonal therapy.[26] PR in combination with ER provides a better indication for treatment response. The percentage of ER-negative, PR-positive tumors from classical assays (1.9%) detected in this study is within the 1.5-6% range reported by others.[27] This particular group of patients is known for or had better outcome from endocrine therapy compared to those negative for both, ER and PR.[27] In addition, the *ER* mRNA-negative, PR-positive tumors (7%) found in the present study are close to the 8.4% reported elsewhere.[8] The discrepancy between ER and PR may simply be explained by false negative results in the methods used. However, some *ER* variants that are unable to bind hormones or to be detected by monoclonal antibodies used in EIA have been found.[1,11] Furthermore, spliced variants containing a deletion at the primer annealing sites will not be detected by RT-PCR assays. These variants of *ER* may play some role in the ER/PR discrepancy.

The ER protein concentration in breast tissue was shown to increase with advancing age and to be related to menopausal status in Asian women[23,28] and in Caucasian women[20,29-30] with breast cancer. *ER* mRNA was also shown to correlate with age[20] but not with menopausal status.[31] In this group of patients, ER protein as well as *ER* mRNA status did not differ significantly for different ages or pre- and post-menopausal status. However, a significant positive relationship between ER protein and age but not for *ER* mRNA and age was found in our patients ($r = 0.24$, $p < 0.0001$)(Fig. 2).

The relationship between the ER status and several clinicopathological parameters has been reported controversially. ER protein was shown to be inversely correlated to tumor size, histological grade and the number of involved lymph nodes by some[8,23,29,32] while other workers did not find such evidence.[30,33] An inverse relationship of *ER* mRNA to tumor size and nuclear differentiation has been reported before.[8,20] Also no association between *ER* mRNA and lymph node invasions was demonstrated.[8,34] A significant negative association between ER protein as well as *ER* mRNA to tumor size was shown in this study. ER protein but not *ER*mRNA was more frequently detected in tumors with high to moderate nuclear differentiation but less frequent in patients having more than 10-nodes invaded.

The ER status is reported to be independent of TNM clinical and pathological stages of breast cancer[28-29] which corresponds to our result. Invasive ductal carcinoma has been shown to be ER-positive more frequently than other histological types.[23] Although this type of tumor is mostly found in our patients (91.5%), we did not find a significant relationship between tumor type and ER protein or *ER* mRNA status. Variations among different studies concerning an association of ER and other prognostic factors for breast cancer may come from ethnic differences, tumor heterogeneity, sample size, measuring methods and cut-off levels of ER concentrations.

A negative relationship between ER protein and p53 or c-erbB-2 oncogene proteins has been reported by our group[35] as well as others.[36-38] *ER* mRNA also differed between p53-positive and p53-negative tumors in this study but the difference was not seen for c-erbB-2.

ER mRNA gave similar and dissimilar information to ER protein. The RT-PCR assay pro-

vides information about *ER* variants but not the EIA. However, whether ER protein or *ER* mRNA relate better to clinical data ramains inconclusive. Quantitation of *ER* mRNA should be an appropriate method for evaluation of ER biology during tumor development. Moreover, it has been confirmed that the ratio of ER protein to *ER* mRNA in ER-positive patients is significantly related to a risk of relapse.[39] We suggest that for a routine clinical use of *ER* mRNA concentrations in breast cancer management, a more sensitive assay for *ER* mRNA detection is required. Furthermore, the concordance of mRNA and protein expression for the *ER* gene should be evaluated in larger groups of breast cancer patients with a longer follow-up time to determine this prognostic relevance.

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