ESR1 gene amplification in an Iranian population with early onset invasive ductal breast carcinoma

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Practice points

- Breast cancer (BC) is a genetically heterogeneous disease in which clinical presentations are varied.
- The product of *ESR1* gene is essential for sexual development and reproductive function and its deficiency is involved in a variety of diseases including breast cancer, endometrial cancer and osteoporosis.
- ER- α expression is considerably higher in premalignant breast disease and malignant BC.
- ER- α high expression status is a good prognostic indicator, and immunohistochemical detection of estrogen receptor expression is routinely used in making decisions on hormonal therapy for BC.
- Gene amplification plays a major role in gene overexpression, therefore, *ESR1* amplification may also play as a crucial prognostic and reliable predictor of clinical response to treatment of BC.
- qPCR is a rapid, cost-effective, highly sensitive and applicable to small tissue samples for quantification of gene dosage.
- Our findings showed that the modified SYBR Green qPCR can be applied for relative quantification of *ESR1* gene copy number with high sensitivity and specificity compared with immunohistochemistry. However, our data need to be confirmed by fluorescence *in situ* hybridization.
- Our data disclosed a significant relationship between *ESR1* amplification and ER- α expression and positive response to tamoxifen.
- *ESR1* amplification also showed a correlation with lower tumor grade and lower tumor stage which suggests a likely prognostic value.

Aim: The study aimed to evaluate a modified SYBR Green qPCR method to study the inter-relations of *ESR1* amplification to other parameters including ER expression status, clinicopathological features and responsiveness to tamoxifen. **Materials & methods:** 35 breast cancer tissues were assessed for *ESR1* amplification by a modified qPCR. **Results:** Amplification of *ESR1* was observed in 31.4% out of 35 samples. *ESR1* amplification and overexpression were significantly correlated (Spearman's Rho = 0.658; $p < 0.001$). *ESR1* amplification was also statistically associated with positive response to tamoxifen ($p = 0.0005$), lower tumor grade ($p = 0.027$) and lower tumor stage ($p = 0.005$). **Conclusion:** Findings showed a positive correlation between ER amplification and ER- α expression and highlighted the potential clinical value of using SYBR Green qPCR to quantify amplification of *ESR1*. *ESR1* amplification could be used as an indicator of positive response to tamoxifen and might have a clinicopathological significance. However, the qPCR data should be confirmed by fluorescence *in situ* hybridization.

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Breast cancer (BC) is a genetically heterogeneous disease in which clinical presentations are varied [1]. Based on the clinical outcome, it is divided into three major subgroups consisting of ER-positive tumors; *HER2*-amplified tumors and triple-negative (basal-like) tumors [2,3]. *ESR1* is located on the chromosome 6q25 and its product, and ER- α regulates the transcription of about 5% of the genome genes [4]. *ESR1* encodes a ligand-activated transcription factor composed of several important domains for hormone binding, DNA binding and activation of transcription [5]. The product of the *ESR1* gene is essential for sexual development and reproductive function, and plays a role in

other tissues such as bone. Involvement of *ESR1* has been reported in a variety of diseases including breast cancer, endometrial cancer and osteoporosis [6]. It has been shown that normal breast epithelial cells express ER- α at a low level and an oscillation expression pattern has been observed throughout the menstrual cycle [7]. However, ER- α expression is considerably higher in premalignant breast disease, and more than two-thirds of breast cancers show estrogen receptor expression at the time of diagnosis. Immunohistochemical detection of estrogen receptor expression is routinely used in making decisions on hormonal therapy for breast cancer [8]. ER- α high expression status is a good prognostic indicator which is associated with longer survival in patients receiving endocrine therapy regimes such as tamoxifen and raloxifene [9].

It is thought that gene amplification plays a major role in the regulation of protein expression. Several amplicons have been reported in different types of cancer which have been related to tumor progression, clinical course of cancer or responsiveness to therapy [10]. *ESR1* amplification may play as a reliable predictor of clinical response to treatment of BC. It is reported that *ESR1* amplification status is an independent and powerful predictor of long-term survival in women with endocrine-responsive early stage breast cancer who received 5 years of tamoxifen [11].

Given the crucial prognostic and predictive information that can be obtained by the analysis of *ESR1* amplification, rapid, accurate and reliable methods for its quantification are required. Immunohistochemistry (IHC) assay is presently used as the method of choice for determination of ER status [12]. Nonetheless, it faces some drawbacks such as being semi-quantitative, low-throughput, subjective, lack of uniformity in reagents, storage and criteria used for scoring, considerable interobserver variability [13].

In addition to the importance of ER- α in response to endocrine therapy, the association of *ESR1* amplification status with patients and clinical parameters and its prognostic/predictive values have not been thoroughly studied to date [14]. Based on a report in 2008, the data gathered from different center revealed that *ESR1* amplification in BC is a rare event of unknown clinical significance [15]. The advent of robust, rapid, sensitive and reproducible quantitative PCR (qPCR) encouraged us to design the present study aimed to quantify the amplification frequency of *ESR1* in an Iranian population with early onset invasive ductal breast carcinoma using a modified SYBR Green qPCR approach, compare the applied qPCR approach with IHC, compare the expression and amplification of ESR and determine the correlation of *ESR1* amplification with the patient and clinical factors and response to tamoxifen therapy.

Materials & methods

Patients

The current study was performed in Tehran, Iran between 2016 and 2017. 35 fresh frozen BC specimens were included in the present study. All the histopathologic diagnoses were performed by pathologists and all the specimens were collected from patients with early onset invasive ductal breast carcinoma. Ten samples taken from a cosmetic breast surgery center were used as the calibrator sample. All cases were histologically classified according to the Bloom–Richardson grading system. Hematoxylin and eosin-stained slides from each case were reviewed to confirm the diagnosis of either invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC). Pathology reports were used to extract the ER- α expression information. Clinicopathological characteristics and IHC data were collected from the pathological and medical documents. The present work was performed under the approval of Research Ethics Committee of Tehran University of Medical Science (Tehran, Iran) and according to the Declaration of Helsinki, a signed consent form was taken from each patient. Assessment of response to tamoxifen was performed based on response evaluation criteria in solid tumors.

DNA extraction & SYBR Green qPCR

We were blind to ER- α expression status before we obtained the relative copy number of *ESR1*. Total DNA was extracted from all the fresh frozen BC tissue sections using QIAamp kit (Hilden, Germany) according to the instruction of manufacturer. Quantity/quality of the extracted DNA was measured by NanoDrop 2000 (MA, USA). Assuming that there are not any differences in the quality and quantity of starting DNA and the PCR efficiency of the target gene, we used $E_{Cp}(\text{gene of interest in calibrator}) - C_p(\text{gene of interest in the case})$ formula to quantify the relative copy number (RCN) of the target gene [16]. Five tenfold serial dilutions of a DNA sample were prepared and the efficiency of each primer was determined according to $E = 10^{-1/\text{slope}}$. Target gene was amplified by SYBR Green-based qPCR using primers: 5'- GCCCCCGATACTCTATTCCG -3' and 5'- CGCTTCGCATTCTTACCTGG -3'. Twenty microliters of PCR reactions containing 10 μ l SYBR Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio, Japan), 1.5 μ l DNA (40 ng/ μ l), 0.5 μ l primer (each, 5 pmol) and 8.5 μ l

Table 1. The qPCR data including Cp values, Δ Ct and $2^{-\Delta Ct}$.

Sample code	Ct value	Δ Ct	$2^{-\Delta Ct}$
T-1	21.65	-1.7	3.25
T-2	20.8	-2.5	5.66
T-3	21.19	-2.16	4.47
T-4	23.51	0.16	0.90
T-5	20.86	-2.49	5.62
T-6	22.65	-0.7	1.62
T-7	22.77	-0.58	1.49
T-8	22.62	-0.73	1.66
T-9	23.73	0.38	0.77
T-10	22.36	-0.99	1.99
T-11	23.52	0.17	0.89
T-12	23.76	0.41	0.75
T-13	21.27	-2.08	4.23
T-14	23.35	0	1.00
T-15	22.82	-0.53	1.44
T-16	23.67	0.32	0.80
T-17	21.11	-2.24	4.72
T-18	22.9	-0.45	1.37
T-19	21.25	-2.1	4.29
T-20	22.54	-0.81	1.75
T-21	21.3	-2.05	4.14
T-22	22.28	-1.07	2.10
T-23	23.75	0.4	0.76
T-24	22.61	-0.74	1.67
T-25	23.91	0.56	0.68
T-26	20.66	-2.69	6.45
T-27	22.64	-0.71	1.64
T-28	23.46	0.11	0.93
T-29	21.36	-1.99	3.97
T-30	20.98	-2.37	5.17
T-31	22.88	-0.47	1.39
T-32	22.25	-1.1	2.14
T-33	23.12	-0.23	1.17
T-34	23.32	-0.03	1.02
T-35	24.06	0.71	0.61

Ct value of pooled normal calibrator was 23.35. As PCR product accumulates, fluorescence signaling increases and rises above background levels at a quantifiable point referred to as the Ct.

double distilled water were run in Rotor–Gene 2000 (Qiagen, Germany) according to the following program: initial heating at 95°C for 30 s, 40 cycles of denaturation at 95°C for 10 s, annealing and extension at 59°C for 30 s. A melting curve was then plotted between 60 and 99°C to the analysis of specific amplification of target region. All quantification analyses were performed based on the cycle of a threshold value (Cp). All reactions were run in duplicates and mean Cp was put into RCN calculation. The RCN of ESR1 was determined by qPCR in tumor sample relative to a normal calibrator reference of CN = 2 (diploid). The qPCR data (Cp values) of the tumor and normal samples are shown in Table 1.

Furthermore, the results of E Cp(gene of interest in calibrator)-Cp(gene of interest in the case) formula were confirmed by using $2^{-\Delta\Delta Ct}$ quantification method, wherein *HBB* and *RPLP0* genes were amplified as internal control and relative-fold differences of target were calculated. A forward primer: GCTTCTGACACAAGTGTGTTCACTAGC and a reverse primer: CACCAACTTCATCCACGTTACAC for a 120 bp fragment of *HBB* and

Table 2. Patients' and tumors' characteristics.

Parameter	Frequency (%)	Parameter	Frequency (%)
Age		ER status (IHC)	
<30	9 (25.7)	Positive	16 (45.7)
>30	26 (74.3)	Negative	19 (54.3)
Tumor size, cm		PR status	
>2	11 (31.4)	Positive	13 (37.1)
<2	24 (68.6)	Negative	22 (62.9)
Tumor stage		ALN metastasis	
I	1 (2.9)	Yes	21 (60)
II	17 (48.6)	No	14 (40)
III	17 (48.6)		
Tumor grade		Family history	
I	9 (25.71)	Positive	14 (40)
II	11 (31.42)	Negative	21 (60)
III	15 (42.85)		

ALN: Axillary lymph node; ER: Estrogen receptor; IHC: Immunohistochemistry; PR: Progesterone receptor.

a forward primer: TGTGGCCTTCTACCTTACTG and reverse primer: TGGGTCTCTTGTCATTTCTC for *RPLP0* was used to amplify the target regions.

Immunohistochemical analysis

Immunohistochemical analysis (IHC) was performed according to previously standardized protocols on an automated IHC platform (Dako Techmate 500) and the ASCO/CAP guidelines for IHC [12]. The following primary antibody and corresponding dilution of ER (clone 1D5; 1:100) were used (DakoCytomation, Glostrup, Denmark). Slides were assessed by quantitative image analysis using the Aperio Image Analysis toolbox (Leica Biosystems, Nussloch, Germany). Staining intensity and percentage of positive nuclei were recorded after manually segmenting tumor from the adjacent stroma. Tumors with ER scores greater than 3 or positive nuclei greater than 1% were considered estrogen receptor positive.

Statistical analysis

The cut-off point of amplification was established by plotting qPCR results compared with IHC data in receiver operating characteristic (ROC) curve. The correlation between applied qPCR and IHC was determined by calculating the Spearman's Rho. The chi-square test was used to analyze associations of gene amplification with clinicopathological characteristics and responsiveness to tamoxifen. All p-values were two-sided and points less than 0.05 were considered statistically significant. SPSS for Windows (version 16.0) was used for statistical analyses.

Results

Patients & clinicopathological data

The oldest and youngest participants were 49 and 16 years old, respectively, and the mean age $\pm$ SD was 37.54 ± 8.83 years. All the collected clinicopathological parameters are demonstrated in Table 2. Regardless the ER- α expression status, 35 IDC tumor samples were investigated for gene amplification of *ESR1* by using qPCR. Flowchart for patients' selection in this study has been illustrated in Figure 1.

Correlation of *ESR1* status with clinicopathological features

Association of *ESR1* status with tumor and patient's characters is shown in Table 3. *ESR1* amplification is statistically associated with ER- α expression ($p < 0.001$), positive response to tamoxifen ($p = 0.0005$), lower tumor grade ($p = 0.027$) and lower tumor stage ($p = 0.005$). However, there are no significant relations between *ESR1* status and age of diagnosis, tumor grade, tumor size, tumor stage, PR status and metastatic status. Spearman's Rho values >0.50 are indicative of a positive relationship between compared groups.

Correlation of qPCR with IHC

qPCR results were compared with IHC data obtained from medical documents of patients. The detection performance or accuracy of applied SYBR Green qPCR test compared with IHC was evaluated using ROC curve analysis. The area under the ROC curve (AUC) is a measure of how well the applied qPCR test can precisely discriminate

Figure 1. Flow chart for patients' selection in this study.

ER: Estrogen receptor; IDC: Invasive ductal carcinoma; IHC: Immunohistochemistry; ILC: Invasive lobular carcinoma; PR: Progesterone receptor; RCN: Relative copy number.

amplified from unamplified conditions. AUC for detection *ESR1* amplification was 0.822 ($p = 0.001$) which is indicative of excellent sensitivity and specificity. Based on the ROC curve analysis, $RCN \geq 3$ was considered as the amplification point of target genes (Figure 2). Spearman's Rho and concordance between qPCR and IHC for detection of *ESR1* status was 0.656 and 82.85% (29/35), respectively. As protein expression is measured in IHC and gene amplification in qPCR, a correlation between qPCR and IHC is also an indication of correlation between gene copy number and protein expression.

Sensitivity, specificity, positive & negative predictive values

The current study was a diagnostic type. Therefore, the sensitivity, specificity, negative predictive value and positive predictive value values were calculated for an applied qPCR method, using IHC as the gold standard. These values were measured to evaluate the performance of applied qPCR to detect the amplification of *ESR1*. Sensitivity, specificity, negative predictive value and positive predictive value were 75, 89.47, 80.95 and 85.71% respectively.

Discussion

The aims of this study were to analyze the frequency of *ESR1* amplification by a modified SYBR Green qPCR, to compare these amplifications with IHC data, to associate these amplifications with clinicopathological features and to correlate the amplification status of *ESR1* with responsiveness to tamoxifen. Our data disclosed that *ESR1* is amplified in 31.4% of the included population affected by early onset invasive ductal breast carcinoma. However, the current study has limitations. The sample size of this study is relatively small. Larger studies are required to determine the percentage of *ESR1* gene amplification in BC definitively. According to our results, the frequency of *ESR1* gene amplification differed than that for reports of other geographical patients with BC. This suggests that the contribution of *ESR1* gene amplification to the pathogenesis and progression of BC is probably different between ethnicities. Technical issues and lack of standards may also contribute to the discrepant findings. Comparison of qPCR results with IHC data showed that applied qPCR could determine the *ESR1* status more rapidly in a time/cost-effective manner with high sensitivity and specificity. Our findings also showed a significant correlation between *ESR1* amplification and ER expression, tamoxifen-responsiveness, lower tumor grade and stage.

Table 3. Patient and tumor characteristics according to ESR1 status.

Patient and tumor characteristics	ESR1 amplified number (%) [†]	ESR1 unamplified number (%)	p-value
All	11 (31.4)	24 (68.8)	–
Age at diagnosis			0.752
>30	4 (28.6)	5 (23.8)	
<30	10 (71.4)	16 (76.2)	
Tumor size, cm			0.298
>2	11 (78.6)	13 (61.9)	
<2	3 (21.4)	8 (38.1)	
Tumor grade			0.027
Grade 1+2	10 (71.4)	7 (33.3)	
Grade 3	4 (28.6)	14 (66.7)	
Tumor stage			0.005
Stage 1+2	12 (85.7)	8 (38.1)	
Stage 3	2 (14.3)	13 (61.9)	
ER status			<0.001
Negative	2 (14.3)	17 (81)	
Positive	12 (85.7)	4 (19)	
PR status			0.886
Negative	9 (64.3)	13 (61.9)	
Positive	5 (35.7)	5 (35.7)	
ALN metastatic			0.778
Negative	6 (42.9)	8 (38.1)	
Positive	8 (57.1)	13 (61.9)	
Family history			0.067
Negative	11 (78.6)	10 (47.6)	
Positive	3 (21.4)	11 (52.4)	
Tam responsiveness [‡]			0.0005
Positive	11 (78.6)	4 (19)	
Negative	3 (21.4)	17 (81)	

The bold information presents data which are statistically significant.

[†]ESR1 amplified number is the percent of samples in which the relative copy number of ESR1 is above the cut-off.

[‡]Tam responsiveness presents the frequency of patient who positively respond to tamoxifen therapy.

ALN: Axillary lymph node; ER: Estrogen receptor; PR: Progesterone receptor; Tam: Tamoxifen.

Given the *ESR1* gene amplification, a large volume of studies on this topic have been published, and a wide range of inconsistent results have been reported. Although there are many reports in which *ESR1* is amplified in 20.6, 4.2, 2, 11.3 and 0%, the reported prevalence of this alteration in breast cancer ranges from 0 to 75%, proposing that *ESR1* copy number analysis is prevented by technical and interpreter issues [4,8,9,14,17–19]. About 70% of breast cancers are ER positive. Gene amplification is the main mechanism for cancer cells to increase the expression of gene products that provide them with a growth or survival advantage. Therefore, amplification may be a potential therapeutic target as same as the example of *ERBB2* and the success of trastuzumab (Herceptin®) for the treatment of Her2-amplified or Her2-overexpressing breast cancers [20]. We found a significant correlation between *ESR1* amplification and ER overexpression. The vast majority of the amplified tumors were also ER-overexpressed which is consistent with previous reports [9,18]. Accordingly, it was found that *ESR1* amplification was significantly related to positive response to tamoxifen, therefore the response to tamoxifen could also rely on *ESR1* amplification. Given the considerable association between *ESR1* amplification and positive estrogen receptor, IHC not only validates our experimental approach but also demonstrates the strong functional importance of *ESR1* gene amplification. Considering the results of the current study and those of the previous report, the mechanism of ER overexpression may be dualistic, consisting of gene amplification as well as activation at the transcriptional level [19,21]. As a result, some cases of inconsistencies could be due to overexpression at the transcription level, not the DNA level, and this could be considered as the limitation of the study when the qPCR and IHC data are compared. Therefore, technical errors might be the reason of low concordance between qPCR and IHC and decrease in sensitivity, specificity, positive and negative predictive values of applied qPCR approach. According to a report, it is more informative to assess the *HER-2/neu* gene amplification than IHC overexpression. Failure to detect fluorescence *in situ* hybridization (FISH)-amplified (IHC-negative) cases would have an adverse effect on the survival of these patients. On the other hand, IHC overexpression tumors without gene amplification appear to belong to a better prognostic group, and failure to detect them would probably not have a negative impact on the survival of these

Figure 2. Receiver-operating characteristic curve. This graphical plot illustrates the diagnostic ability including sensitivity and specificity of applied SYBR Green qPCR approach. Relative copy number ≥ 3 was set for ROC analysis, wherein, $AUC = 0.822$, $p = 0.001$ and 95% CI: 0.672–0.973. These data are indicative of an excellent diagnostic ability of the applied qPCR in comparison to immunohistochemistry. The blue line represents the plot of sensitivity versus 1-specificity and the green line is a tangent line whose slope at a cut-point gives the likelihood ratio for that value of the test.

ROC: Receiver-operating characteristic.

women [22]. The same scenario may also be the reason of nonresponsiveness of ER- α -positive tumors without *ESR1* amplification to tamoxifen or other antiestrogen therapy. However, this is just a hypothesis and must be evaluated, ideally in an experimental setting. Unfortunately, recurrences still do occur in 30–40% of ER positive breast cancer patients, and *ESR1* or other biomarkers amplification may be due to the etiology of nonresponsiveness to tamoxifen. Sanaz Tabarestani and colleagues showed that *CCND1* amplification may serve as a useful biomarker for hormone responsiveness [23].

Microarray studies on tumors and breast cancer cell lines have shown that 40–61% of overexpressed genes have also been genetically amplified and the findings have provided evidences that widespread DNA copy number alteration can lead directly to global deregulation of gene expression [24]. The main purpose of the current study was to compare *ESR1* amplification with its expression status and to show how efficiently qPCR can determine *ESR1* amplification as a complementary tool to other techniques such as FISH. A broad range of techniques such as array comparative genomic hybridization, chromogenic in situ hybridization (CISH), FISH and qPCR has been applied for quantitation of ESR copy number [4,9,15,25]. Given the critical role of ER expression for breast cancer therapy and the importance of gene amplification in overexpression, need to a rapid, cost-benefit and accurate method for *ESR1* profiling is highlighted. Although each technique has its advantages and disadvantages, a modified qPCR approach was developed to determine the *ESR1* copy number. The SYBR Green and TaqMan assays are usual chemistries for real-time PCR. Owing to its simple design, easy setup, and low cost, SYBR Green is predominantly used for the detection and quantification of nucleic acids. Absolute and relative quantification are two different PCR-based methods of presenting quantitative gene copy number. Absolute quantification is based on the calculation of the copy number of a target gene by relating the PCR signal to a standard curve. However,

relative gene copy number quantification presents the data of target gene relative to some calibrator or internal control gene. According to our knowledge, absolute quantification or relative quantification based on hybridization probes has previously been applied to gene copy number quantification. Nonspecific binding of the SYBR Green dye to the nontarget double-strand DNA molecule can lead to false-positive results. However, any limitations in the specificity of dsDNA dyes can be overcome by using postamplification melting curves or choosing high fluorescence acquisition temperatures during amplification and good primer design and optimization of real-time PCR. The most famous qPCR strategies are based on hybridization probes, but they are still expensive and hard to be optimized.

In contrast with previously reported qPCR methods which have been based on hybridization probes, a modified SYBR Green-based qPCR approach was used to relatively quantify the copy number of *ESR1*. The common method of relative quantification is based on $2^{-\Delta\Delta C_t}$ formulae. Assuming that there are not any differences in the quality and quantity of starting DNA and the PCR efficiency of the target gene, a modified SYBR Green qPCR based on $2^{Cp(\text{gene of interest in calibrator})-Cp(\text{gene of interest in the case})}$ formula was used to the RCN of *ESR1*. In this approach, internal reference gene was not assessed so that the number of required reactions is decreased considerably and consequently time and cost. Due to sensitivity to contamination with normal stromal cells, DNA extraction was performed from biopsied cells of microdissected tissue sections containing more than 85% tumor cells. Besides, the accuracy of the applied formula was confirmed using the common method of relative quantification, $2^{-\Delta\Delta C_t}$, in which *HBB* and *RPLP0* genes were amplified as the internal single copy reference genes. The relationship between $2^{-\Delta C_t}$ and $2^{-\Delta\Delta C_t}$ methods in determining the *ESR1* copy number of is illustrated in Figure 3. The linear relationship between the results of the two methods indicated the accuracy of the modified $2^{-\Delta C_t}$ approach used in this study. Although FISH is the gold standard way to profile gene copy number and is more objective and quantitative than immunohistochemical scoring, it is more expensive, fluorescence signals fade within a few weeks and the FISH assay can be interpreted only by well-trained personnel.

Our data revealed 82.85% concordance and significant correlation between qPCR and IHC; therefore the data presented suggest that the *ESR1* amplification status might be determined by qPCR and could be used complementarily in addition to IHC to potentially compensate limitations of IHC, and in making decisions on hormonal therapy for breast cancer. Concordance between qPCR and IHC has been reported in different kinds of literature in which the value of correlation has been varied in a range of 80–95% [26]. Consistent with our findings, IHC for ER and PR revealed a high concordance with RT-qPCR (Spearman's $\rho = 0.82$; $p < 0.0001$) in a recent study. However, RT-qPCR displayed a broader dynamic range and higher sensitivity than IHC [19].

Association of *ESR1* amplification status with patients and tumors' characteristics was also assessed as a further aim. As we performed the current work on a specific type of breast cancer, early onset invasive ductal carcinoma, this essential inclusion criterion for the study population led to the limitation of sample size. However, this point could be considered as the limitation of the study. Correlation of *ESR1* amplification with clinicopathological parameters has been studied. Our findings showed that *ESR1* amplification is considerably related to lower tumor grade and stage. These findings would be consistent with a very early, if not initializing, a role for ER amplification for a subset of breast cancers. Thus, it might be concluded that *ESR1* amplification status shows a useful prognostic marker and suggests that *ESR1* amplification may be a common mechanism in individuals with BC. It is reported that epigenetic signature within the *ESR1* gene CpG island is changed in a series of nonmalignant and preneoplastic breast tissues [27]. Although these findings have been confirmed in a small number of reports, there are a variety of studies with contradictory results in which *ESR1* status has been linked with higher tumor grade, stage, size, nodal metastasis and poorer outcome [4,8,9,14,28]. The prognostic or predictive power of the *ESR1* amplification status determined by qPCR was compared with the prognostic or predictive power of the ER- α status determined by IHC. Data showed that ER- α status was significantly associated with tumor grade ($p = 0.005$), tumor stage ($p = 0.004$) and positive response to tamoxifen ($p = 0.000000024$).

We are aware of several limitations of this study, including small sample size, qPCR sensitivity to DNA quality and quantity, falling qPCR results between integers and therefore more difficult interpretation, sensitive to finding a true cut-off point of amplification, qPCR sensitivity to mosaicism, and contamination with a large number of normal stromal cells. To avoid these limitations as much as possible, different filtering criteria and steps are proposed to be considered in the process of work design including microdissection of tumor tissue to cut the area containing >85% cancer cells, primer design and in silico quality control, empirical validation of primers, preparation of gDNA dilution series for qPCR assay evaluation and melt curve analysis. Also, the study of a larger population might result in more validated data. The accuracy of the applied formula is also suggested to

Figure 3. The relationship between $2^{-\Delta\Delta ct}$ (modified qPCR approach) and $2^{-\Delta\Delta ct}$ (conventional qPCR approach) methods in determining the copy number of the *ESR1* gene. In this chart, the linear relationship between the results of the two methods indicates the high accuracy of the modified $2^{-\Delta\Delta ct}$ approach used in the current study. The numbers at the vertical axes represent the relative copy number of *ESR1* determined by the modified qPCR approach and the numbers at the horizontal axes represent the relative copy number of *ESR1* determined by the conventional qPCR approach. R Sq Linear is 0.92.

be confirmed using FISH and this could be mentioned as another limitation of the study. Currently, emphasis must be put on standardization of procedures, internal and external quality control assessment, and competency evaluation of already existing methods to ensure accurate, reliable and clinically meaningful test results. However, development of new cost/time benefit, robust and accurate diagnostic assays is also required to be encouraged.

Conclusion

The present study highlights the potential clinical value of using SYBR Green qPCR to quantify amplification of *ESR1*. Although qPCR provides quick and robust results, a standardized and validated definition of a cut-off point of amplification needs to be investigated before its potential use in daily practice. The accuracy of the applied qPCR is also suggested to be confirmed using FISH. Our data disclosed a significant relationship between *ESR1* amplification and ER- α expression, positive response to tamoxifen, lower tumor grade and lower tumor stage. However, there were inconsistencies between *ESR1* amplification and IHC data and determination of the *ESR1* amplification status may not be confidently suggested to be an alternative to ER- α status determined by IHC. Furthermore, more studies with larger sample size are suggested to reproduce and confirm our findings. The aim of strategically BC management would be achievable through unmasking the functional event at tumor level. Nevertheless, by considering the complementary dual assay such as expression status of *ESR1* and *Her2-neu*, could

be translatable to the target- and endocrine-based therapy. Combinational therapy also relies on expression mode of *ESR1* and Her2-neu including Er+/Her2-, Er+/Her2+ for updating the approach of clinical management for BC patients including the metastatic BC. Besides, exploring the role of the available biomarker(s) for the prevention, assessment, and management guidelines of breast cancer can be considered as a future perspective.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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