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Detection of *CCND1*, *C-MYC*, and *FGFR1* amplification using modified SYBR Green qPCR and FISH in breast cancer

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Background/aim: The aims of this study were to detect *CCND1*, *C-MYC*, and *FGFR1* amplification using qPCR, confirmation with FISH, and to further assess their clinicopathological relevance.

Materials and methods: Thirty-five breast tumor samples were analyzed for amplification of the selected genes using modified SYBR Green qPCR. The accuracy of the qPCR was assessed by FISH as a gold-standard method.

Results: *CCND1*, *C-MYC*, and *FGFR1* amplifications were observed in 34.28%, 28.57%, and 17.14% of the 35 samples, respectively. qPCR results were significantly confirmed by FISH and qPCR and FISH showed excellent correlation (P = 0.000). *CCND1* amplification with tumor stage (P = 0.044), positive metastatic status (P = 0.042), positive family history (P = 0.042), and *C-MYC* status (P = 0.005); *C-MYC* amplification with tumor size (P = 0.021), tumor grade (P = 0.018), tumor stage (P = 0.032), and *FGFR1* status (P < 0.000); and *FGFR1* amplification with tumor size (P = 0.041) and positive *ER* status (P = 0.042) were statistically associated.

Conclusion: Our findings revealed that the applied qPCR approach could precisely quantify the relative gene copy number. More studies with a larger sample size are suggested to confirm the clinicopathological value of *CCND1*, *C-MYC*, and *FGFR1* amplification.

Key words: Breast cancer, fluorescent in situ hybridization, gene amplification, gene copy number, real-time polymerase chain reaction

1. Introduction

Cancer originates from the interaction of the environment and accumulative genetic changes such as mutations, copy number variations (CNVs), and epigenetic alterations. Gene amplification (GA) is one type of CNV known as the increase of a defined cytogenetic region of chromosomes called amplicons. CNVs are a common event in solid tumors and GA is the main mechanism that leads to the activation of protooncogenes (1). Chromosomal positions at 1q, 8p12, 8q24, 11q13, 12p13, 16p13, 7q12-21, and 20q13, and several target oncogenes including *ERBB2*, *MYCL1*, *MYCN*, *REL*, *EGFR*, *FGFR1*, *CCND1*, *TOP2A*, and *C-MYC*, have been the most prominent and frequent amplicons identified in breast cancer (BC). There are many common amplification-activated human oncogenes identified in different cancers (2–4).

Cyclin D1 is encoded by the *CCND1* gene and has a prominent role in the progression of a cell through the G1 phase of the cell cycle by complexing with cyclin-dependent kinases *CDK4* and *CDK6*. *CCND1* amplification has been identified in a variety of tumors, among which 10%–15% of human primary breast cancers have shown amplification in the 11q13 chromosomal region (5). There are numerous studies that ascertain whether *CCND1* amplification might be associated with clinicopathological variables and clinical outcome as a biological marker in BC; however, the results are contradictory (6). *C-MYC* encodes a transcription factor that regulates cell growth, proliferation, metabolism, differentiation, and apoptosis. It is amplified in the range of 10%–16% in BC. Although it is reported to be related to the risk of relapse and death, its prognostic value is not completely clear (7). The fibroblast growth factor receptor 1 gene (*FGFR1*) provides information for producing a tyrosine kinase receptor and is amplified in 9% to 15% of BC cases, thought to be correlated with poor prognosis in BC (8).

Given the crucial prognostic and predictive information that can be obtained by the analysis of *CCND1*, *C-MYC*, and *FGFR1* amplification, rapid, accurate, and reliable methods for quantification are required. Depending on the required resolution, rapidity, cost, application, etc., the method of choice for gene quantification could be different, but each technique has some advantages and disadvantages. Although array and probe-based methods

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are powerful tools for genome scanning or simultaneous study of multiple genetic markers, they may be complicated and difficult to be automated, validated, standardized, and interpreted. Therefore, they may not be appropriate options for clinical applications and to only assess a small number of target genes (9–11).

qPCR can, however, determine the overall amplification of genes accurately. It is often homogeneous and reduces time, the risk of contamination, and manual errors. qPCR is objective, fast, versatile, sensitive, specific, and time- and cost-effective and can be performed on a limited number of DNA samples. Gene dosage is measured by two approaches in qPCR: fluorescent dyes and intercalating dyes. Regardless of the approach, fluorescence doubles with every cycle of PCR, and the amount of gene copy can be determined based on the number of cycles required to rise above a specified threshold level of fluorescence. Double-stranded DNA-specific binding dye-based detection systems are more cost-effective, simpler to design, and easier to set up than hydrolysis probes, molecular beacons, and dual hybridization probe-based strategies (12,13).

The aims of this study are fourfold: to assess the accuracy of SYBR Green qPCR for detection of gene amplification; to analyze the amplification of *CCND1*, *C-MYC*, and *FGFR1* amplification in an early-onset BC population using SYBR Green qPCR; to analyze the correlations between *CCND1*, *C-MYC*, and *FGFR1* amplifications and clinicopathological features; and to define the correlations of *CCND1*, *C-MYC*, and *FGFR1* amplifications with each other.

2. Materials and methods

2.1. Sample preparation

Thirty-five fresh frozen BC tissue specimens were used in the present study. All the participating patients in the study had early-onset invasive ductal breast carcinoma. All the histopathological diagnoses were performed by pathologists. DNA and cell suspensions were used for qPCR and FISH analysis, respectively. Ten samples taken from a cosmetic breast surgery center were used as the calibrator. The present work was performed under the approval of the Research Ethics Committee of Tehran University of Medical Science (Tehran, Iran) and according to the Declaration of Helsinki, and a signed consent form was received from each patient.

2.2. DNA extraction and SYBR Green qPCR

Total DNA was extracted from microdissected fresh frozen BC specimens using a QIAamp kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer. A modified $E^{\text{Cp (gene of interest in calibrator)} - \text{Cp (gene of interest in case)}}$ formula was used to determine the relative copy number (RCN) of selected genes based on the fact that there are no differences in the quality and quantity of starting material and there is no difference in PCR efficiency between target genes (14). Therefore, sections of tumor tissues containing >80% cancerous cells were dissected for downstream qPCR and FISH analysis. All extracted DNAs were assessed for quantity and quality with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples of 40 ng/μL, 260/280 = 1.8–1.9, were provided for qPCR assay. Five 10-fold serial dilutions were prepared and the efficiency of each primer was determined according to E = 10 – 1/slope. RCN was measured by SYBR Green-based qPCR using the following primers: 5'-GATTGGAGGCACACGTCTCA-3' and 5'-GCTCAGCTACGTTGGTCACT-3' for *CCND1*, 5'-AAAAGTGGGCGGCTGGATAC-3' and 5' AGGGATGGGAGGAAACGCTA-3' for *C-MYC*, and 5'-CCGCTCCCTAAACTTGCTGA-3' and 5'-AGGAATGAGACGGGATTGCG-3' for *FGFR1*. PCR reactions of 10 μL were run in a Rotor-Gene 2000 (QIAGEN) using SYBR Premix Ex Taq (Tli RNaseH Plus, Takara Bio, Shiga, Japan) according to the following program: initial heating at 95 °C for 30 s, and 40 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. Melting curve analysis from 60 to 99 °C was also run to assess specific amplification of target regions in each sample. All quantification analyses were accomplished based on the cycle of a threshold value (Cp). All reactions were run in duplicate and mean Cp was put into RCN calculations. RCNs of <0.6, 0.6–1.5, 1.5–3, and >3 were considered as loss, normal, gain, and amplified, respectively. Those samples with RCN of >2.5 were analyzed by FISH as the gold-standard method.

2.3. Fluorescence in situ hybridization (FISH) analysis

XL *CCND1*, XL MYC amp, and XL *FGFR1* probe kits (Metasystems, Altlußheim, Germany) were used for FISH analysis of the target genes. In summary, tissue samples of 10 μm were treated with pepsin for 90 min at 37 °C and were fixed in 1:3 acetic acid:methanol solution. Specimens were spotted onto clean microscopic slides, air-dried, and incubated in 2X saline sodium citrate (SSC) buffer (pH 7–7.5) containing 0.9% Tween-20 (pH 7) at 37 °C for 30 min; dehydrated in 70%, 80%, and 95% ethanol (each for 1 min at room temperature); washed with ddH2O; and left to air-dry. The next steps were conducted according to the manufacturer's instructions with a little modification whereby the codenaturation time was increased to 6 min and the posthybridization time of washing with 0.4X SSC was increased to 5 min. The slides were counterstained with DAPI/antifade and analyzed with a microscope (Leica, Wetzlar, Germany) after 10 min. The signal of the target gene was scored in 200 nuclei and the ratio of signals/number of scored cells was measured. Ratios of >2.2 were considered as signifying gene amplification.

2.4. Statistical analysis

The cut-off point for assigning negative and positive status for SYBR Green qPCR was established by plotting receiver operating characteristic (ROC) curves. The correlation between methods was determined by calculating the Spearman correlation coefficient. The chi-square test or Fisher exact test was used when comparing gene amplification frequencies between groups and analyzing associations of gene amplification with clinicopathological characteristics. $P < 0.05$ was considered statistically significant. All P-values reported are two-sided. The statistical software package used for these analyses was SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Samples

Thirty-five female patients with early-onset BC were enrolled in this study. The population of the study was in normal distribution; the maximum and minimum participant ages were 16 and 49 years old, and mean ± SD was 37.54 ± 8.83 years.

3.2. Copy number quantification by SYBR Green qPCR

Gene copy number (GCN) measurement of *CCND1*, *C-MYC*, and *FGFR1* was performed for 35 fresh frozen BC specimens using a double-stranded DNA-specific binding dye (SYBR)-based qPCR strategy. Percentages of samples showing CNRs of >3, 1.5–3, 0.6–1.5, and <0.6 are shown in Figure 1. Samples with RCN of >2.5 were also analyzed by FISH for assessing the accuracy of qPCR results. The detection performance or accuracy of the applied SYBR Green qPCR test was compared to FISH in order to discriminate amplified from nonamplified conditions using ROC curve analysis. The area under the ROC curve (AUC) is a measure of how well a test can precisely discriminate these conditions. AUC values for detection of *CCND1*, *C-MYC*, and *FGFR1* amplification were 0.90, 0.85, and 0.83, respectively. Based on these findings, RCN of ≥3 was considered as the amplification cut-off point of the target genes.

3.3. FISH analysis

As indicated, samples with RCN of >2.5 were analyzed by FISH in order to confirm the accuracy of the applied SYBR Green qPCR and identify the true amplification cut-off point. Comparison of the results of applied methods with ROC curve analysis showed RCN of ≥3 as the true amplification point. However, FISH analysis showed a few inconsistencies. One sample for *CCND1* and one sample for *FGFR1* were unamplified based on FISH. Also, one sample with RCN of 2.75 was amplified for *C-MYC* by FISH analysis. There were a total of three samples with an ambiguous score that were retested and finally 2 samples were scored as normal and one sample was scored as amplified. The highest to lowest correlation rate between the two applied methods was observed for *CCND1* (0.815; $P < 0.000$), *C-MYC* (0.775; $P < 0.000$), and *FGFR1* (0.720; $P < 0.000$) (Table 1).

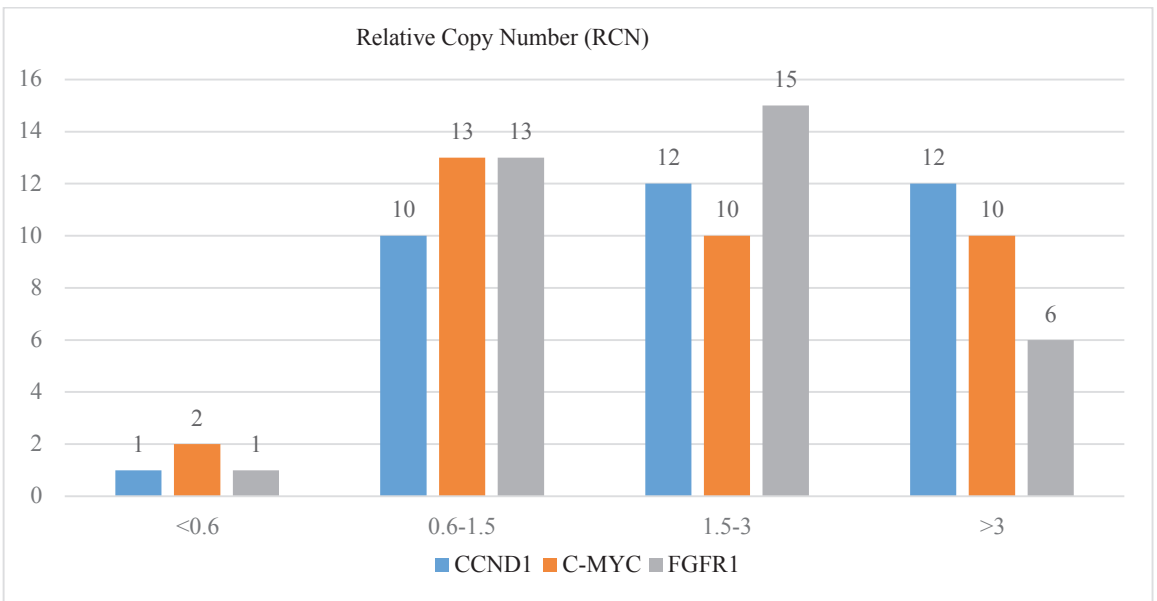

Figure 1. Percentage of BC samples with RCNs* of <0.6, 0.6–1.5, 1.5–3, and >3 measured using qPCR. *Relative copy numbers. RCNs of <0.6, 0.6–1.5, 1.5–3, and >3 were considered as loss, normal, gain, and amplified, respectively.

Table 1. Comparison of qPCR-FISH for detection of amplification in CCND1, C-MYC, and FGFR1 genes.

	CCND1-FISH		P-value	Correlation coefficient*
CCND1-qPCR Unamplified Amplified	Normal (%) 21 (95.5) 1 (4.5)	Amplified (%) 2 (15.4) 11 (84.6)	<0.000	0.815
	C-MYC-FISH			
C-MYC-qPCR Unamplified Amplified	Normal (%) 21 (100) 0 (0)	Amplified (%) 4 (28.6) 10 (71.4)	<0.000	0.775
	FGFR1-FISH			
FGFR1-qPCR Unamplified Amplified	Normal (%) 27 (96.4) 1 (3.6)	Amplified (%) 2 (28.6) 5 (71.4)	<0.000	0.720

*Spearman correlation (Spearman's rho) between the applied methods to detect *CCND1*, *C-MYC*, and *FGFR1* amplification; >0.70 demonstrates a strong positive linear relationship.

3.4. Association of *CCND1*, *C-MYC*, and *FGFR1* status with clinicopathological features

The clinical and tumor characteristics of the included samples according to *CCND1*, *C-MYC*, and *FGFR1* status are shown in Tables 2 and 3. *CCND1* amplification is statistically associated with tumor stage 1+2 (P = 0.044), positive nodal metastasis (P = 0.042), positive family history (P = 0.02), and *C-MYC* status (P = 0.005); however, *CCND1* is not significantly related to age at diagnosis, tumor grade, tumor size, *ER* status, or *FGFR1* status (Table 2). *C-MYC* status was significantly related to larger tumor size (P = 0.021), higher tumor grade (P = 0.018), higher tumor stage (P = 0.032), and *FGFR1* status (P < 0.000). There was no significant correlation between *C-MYC* status and all the other clinicopathological parameters (Table 2). *FGFR1* amplification was statistically correlated with tumor size (P = 0.041), *C-MYC* status (P < 0.000), and positive *ER* status (P = 0.042). Association of *FGFR1* status with other clinicopathological characteristics was not significant (Table 3).

4. Discussion

Pathogenic variations in GCNs are a hallmark of cancer that frequently occur in the process of cancer development. Gene amplification is a major strategy of oncogene overexpression in malignant tumors (15–17). Thus, DNA-based copy number detection methods could be helpful for the better management of cancer including therapeutic decisions and prognosis detection. Due to the greater stability of DNA compared to RNA and proteins, gene amplification detection assays may therefore be optimally appropriate for diagnostic applications (16).

Although there are different methods for measuring the GCN, a fast, reproducible, and cost-effective method is required. qPCR has individual advantages including rapidity, cost-effectiveness, and accuracy. Two different PCR-based methods of presenting quantitative GCN exist: absolute and relative quantification. Absolute quantification calculates the copy number of the gene usually by relating the PCR signal to a standard curve. Relative GCN quantification presents the data of the gene of interest relative to some calibrator or internal control gene. Based on the authors' knowledge, absolute quantification or relative quantification based on hybridization probes has previously been applied to GCN quantification. qPCR techniques based on hybridization probes are still expensive and are hard to optimize. In the present study, modified SYBR Green-based relative quantification was utilized for measuring the GCNs of three crucial oncogenes, *CCND1*, *C-MYC*, and *FGFR1*, in early-onset BC samples. The modified $E^{-\Delta Ct}$ formula was used for measuring RCNs of the target genes in tumor samples compared to the calibrator, assuming that there are no differences in the quantity of starting material and no difference in PCR efficiency between target genes in DNA samples extracted from tumor and normal calibrator tissues. To achieve a highly specific, sensitive, and efficient qPCR reaction, different criteria and steps including microdissection of tumor tissue to obtain the area containing >80% cancer cells, selection of regions, primer design and in silico quality control, empirical validation of primers, and standard curve and melting curve analysis were considered in the process of this work's design. The

Table 2. Patient and tumor characteristics according to *CCND1* and *C-MYC* status.

	*CCND1** Amplified number (%)	*CCND1* Unamplified number (%)	P-value	*C-MYC* Amplified number (%)	*C-MYC* Unamplified number (%)	P-value
All	12 (34.3)	23 (65.7)		10 (28.6)	25 (71.4)	
Age at diagnosis >30 years <30 years	 3 (25) 9 (75)	 6 (26.1) 17 (73.9)	0.944	 2 (20) 8 (80)	 7 (28) 18 (72)	0.625
Tumor size, cm >2 <2	 6 (50) 6 (50)	 18 (78.3) 5 (21.7)	0.087	 4 (40) 6 (60)	 20 (80) 5 (20)	0.021
Tumor grade Grade 1+2 Grade 3	 1 (8.3) 11 (91.7)	 9 (39.1) 14 (60.9)	0.056	 0 (0) 10 (100)	 10 (40) 15 (60)	0.018
Tumor stage Stage 1+2 Stage 3	 14 (60.9) 9 (39.1)	 3 (25) 9 (75)	0.044	 2 (20) 8 (80)	 15 (60) 10 (40)	0.032
*ER** status Positive Negative	 5 (41.7) 7 (58.3)	 11 (47.8) 12 (52.2)	0.728	 4 (40) 6 (60)	 15 (60) 10 (40)	0.283
*PR** status Positive Negative	 4 (33.3) 8 (66.7)	 9 (39.1) 14 (60.9)	0.736	 6 (60) 4 (40)	 16 (64) 9 (36)	0.825
Metastatic status Positive Negative	 10 (83.3) 2 (16.7)	 11 (47.8) 12 (52.2)	0.042	 2 (20) 8 (80)	 12 (48) 13 (52)	0.125
Family history Negative Positive	 10 (83.3) 2 (16.7)	 11 (47.8) 12 (52.2)	0.042	 5 (50) 5 (50)	 16 (64) 9 (36)	0.445
C-MYC status Normal Amplified	 5 (41.7) 7 (58.3)	 20 (87) 3 (13)	0.005			1
*FGFR1** status Normal Amplified	 8 (66.7) 4 (33.3)	 21 (91.3) 2 (8.7)	0.066	 4 (40) 6 (60)	 25 (100) 0 (0)	<0.000
CCND1 status Normal Amplified			1	 3 (30) 7 (70)	 20 (80) 5 (20)	0.005

**CCND1*: Cyclin D1, *ER*: estrogen receptor, *PR*: progesterone receptor, *FGFR1*: fibroblast growth factor receptor 1.

accuracy of the applied formula was confirmed using the common method of relative quantification, $2^{-\Delta\Delta Ct}$, in which the *HBB* (hemoglobin, beta) and *RPLP0* (ribosomal protein 109 lateral stalk subunit P0) genes were amplified as internal controls. Comparison of the two methods of qPCR-relative quantification confirmed the high accuracy of the modified $E^{-\Delta Ct}$ formula. Although probe-based or absolute quantification relating the PCR signal to a standard curve has been used to detect GCNs (18,19), to the best of the authors' knowledge, relative quantification to detect RCNs by relating the PCR signal of the target genes in one group to another is applied for the first time in human tumor samples in the current study.

Additionally, the accuracy of the applied SYBR Green qPCR was also assessed by FISH as the gold standard (Figure 2). qPCR results in comparison to FISH results

Table 3. Patient and tumor characteristics according to *FGFR1* status.

	FGFR1 Amplified number (%)	*FGFR1* Unamplified number (%)	P-value
All	6 (17.1)	29 (82.9)	
Age at diagnosis >30 years <30 years	 2 (33.3) 4 (66.7)	 7 (24.1) 22 (75.9)	0.639
Tumor size, cm >2 <2	 2 (33.3) 4 (66.7)	 22 (75.9) 7 (24.1)	0.041
Tumor grade Grade 1+2 Grade 3	 0 (0) 6 (100)	 10 (34.5) 19 (65.5)	0.089
Tumor stage Stage 1+2 Stage 3	 1 (16.7) 5 (83.3)	 16 (55.2) 13 (44.8)	0.086
ER status (IHC) Negative Positive	 1 (16.7) 5 (83.3)	 18 (62.1) 11 (37.9)	0.042
PR status (IHC) Negative Positive	 5 (83.3) 1 (16.7)	 17 (58.6) 12 (41.4)	0.254
Metastatic status Negative Positive	 2 (33.3) 4 (66.7)	 12 (41.4) 17 (58.6)	0.714
Family history Negative Positive	 2 (33.3) 4 (66.7)	 19 (65.5) 10 (34.5)	0.143
CCND1 status Normal Amplified	 2 (33.3) 4 (66.7)	 21 (72.4) 8 (27.6)	0.066
C-MYC status Normal Amplified	 0 (0) 6 (100)	 25 (86.2) 4 (13.8)	<0.000

were plotted in the ROC curve to illustrate the qPCR diagnostic ability, including sensitivity and specificity, for detection of RCNs of selected genes. The graphs in Figure 3 show three ROC curves representing excellent (*CCND1*) and good (*C-MYC* and *FGFR1*) ability of the applied qPCR. The accuracy of the qPCR assay was measured by the AUC, which was 0.90, 0.857, and 0.839 for *CCND1*, *C-MYC*, and *FGFR1*, respectively. Correlation coefficients between *CCND1*-qPCR, *C-MYC*-qPCR, and *FGFR1*-qPCR and FISH were 0.815, 0.775, and 0.720 ($P < 0.000$), respectively (Figure 2; Table 1).

These findings indicate that the applied qPCR approach has a strong linear relationship with FISH and can relatively quantify the copy number of target genes from only a few biopsy cells with high sensitivity and specificity. However, this approach might face some limitations, including sensitivity to DNA quality and quantity, more difficult interpretation, sensitivity to finding a true cut-off point of amplification, sensitivity to mosaicism, contamination with a large number of normal stromal cells, and methodological and experimental errors. One sample with RCN of >3 was negative in FISH for *FGFR1* and one sample with RCN of 2.75 was positive in FISH for *C-MYC*, which could be due to these mentioned limitations.

Association of selected genes status with patient and tumor characteristics was also assessed as a further

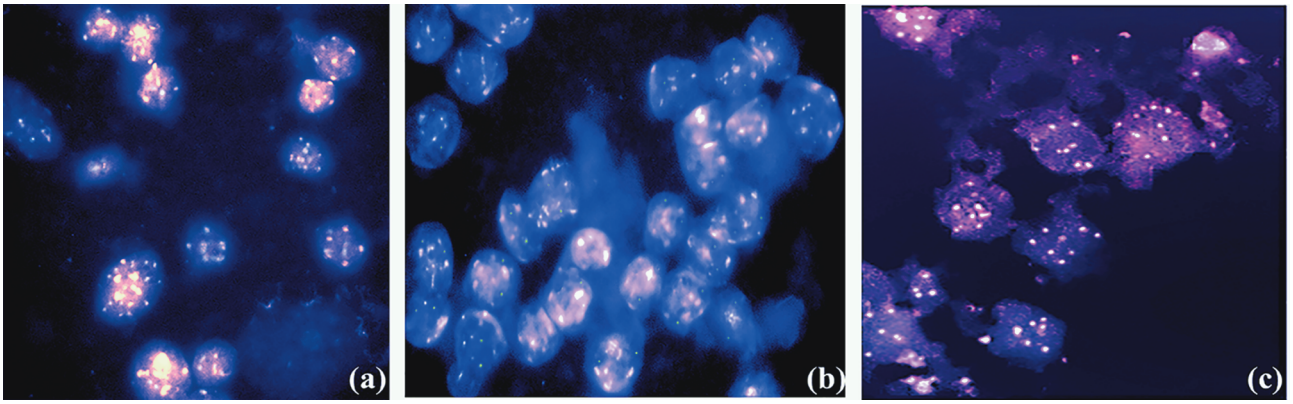

Figure 2. Confirmation of *CCND1*, *C-MYC*, and *FGFR1* gene amplification by fluorescence in situ hybridization (FISH). a) Gene amplification of *CCND1* in BC tumor sample 1. A tumor with multiple individual *CCND1* signal-type amplifications (green-orange). *CCND1* relative copy number in BC tumor 1 is 11.15 based on qPCR. 1000×. b) Gene amplification of *C-MYC* in BC tumor sample 14. A tumor with clustered and multiple individual *HER2* signal-type amplification (*C-MYC* signal is displayed in orange and green signal represents centromere region). *C-MYC* relative copy number in BC tumor 14 is 4.56 based on qPCR**.** 1000×. c) Gene amplification of *FGFR1* in BC tumor sample 23. A tumor with multiple individual *CCND1* signal-type amplifications (green-orange). *C-MYC* relative copy number in BC tumor 23 is 5.89 based on qPCR. 1000×.

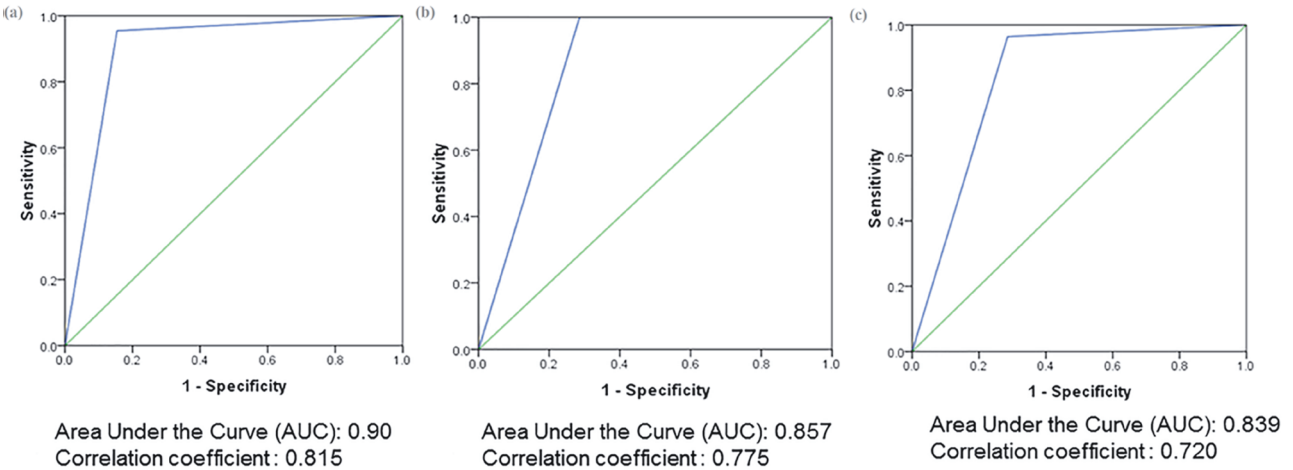

Figure 3. Receiver operating characteristic (ROC) curves. These graphical plots illustrate the diagnostic ability, including the sensitivity and specificity, of the applied SYBR Green qPCR approach. Correlation of qPCR and FISH was calculated by statistical Spearman correlation test: a) ROC curve of qPCR to detect RCN of *CCND1*, b) ROC curve of qPCR to detect RCN of *C-MYC*, c) ROC curve of qPCR to detect RCN of *FGFR1*.

aim. As we performed the current work on a specific type of BC, early-onset invasive ductal carcinoma, the essential inclusion and exclusion criteria for the study population led to the limitation of the sample size. This could be considered as a limitation of the study. *CCND1* amplification has been observed in 10%–27% of BC cases (20). Prognostic properties of *CCND1* in BC have been reported in several studies with conflicting results (5,6). It has also been shown that *CCND1* amplification preferentially occurs in estrogen receptor-positive BC and *CCND1* amplification has been suggested as being associated with resistance to tamoxifen therapy (21). In the present study, *CCND1* amplification was observed in 34.3% (12/35) of samples. Our data showed that *CCND1* amplification might be a poor prognostic biomarker and a significant relation was found between its status and age at diagnosis, tumor grade, tumor size, *ER* status, positive family history, and *FGFR1* status. It could be concluded from the association with positive family history that either inheritance of a pathogenic mutation in key genes such as BRCA1/2 and P53 may drive amplification of *CCND1* or this connection may be due to small sample size and

is not the true relation. Correlation between *CCND1* and *FGFR1* could be explained by their crosstalk in cell growth pathways and tumorigenesis. It has been demonstrated that *CCND1* and *FGFR1* coamplification results in the localization of 11q13 and 8p12 sequences in breast tumor nuclei (22). C-amplification of *CCND1* and *C-MYC* was observed in 7 amplified samples, which probably reflects the possible synergistic contribution to tumorigenesis.

C-MYC is a pivotal regulator of up to 15% of human genes (23). Gene amplification is one of the common mechanisms of *C-MYC* deregulation in BC and it is also amplified among a variety of solid cancers (24). Although *C-MYC* amplification has been observed in a range of 8%–37% in BC, *C-MYC* amplification in the present work was 28.6% (16,25). The overwhelming majority of studies have demonstrated the association of *C-MYC* amplification with tumor grade, lymph node metastasis, negative progesterone receptor status, postmenopausal status, the risk of relapse and death, and poor prognosis (23). Our findings revealed a considerable association between *C-MYC* status and larger tumor size, higher tumor grade, higher tumor stage, and *FGFR1* status that could confirm the poor prognostic value of *C-MYC* amplification. It could also be concluded that *C-MYC* may play a role in an *FGFR1*-dependent model of BC tumorigenesis.

FGFR1 is located at cytogenetic location 8p11.23, which initiates a cascade signaling pathway and triggers mitogenesis and differentiation. *FGFR1* was one of the first genes found to be amplified in 10% of BC patients; however, our data showed 17.14% (5/35) amplification. *FGFR1* amplifications are reported to be associated with anchorage-independent proliferation, endocrine therapy resistance, early relapse, and poor survival, particularly in ER-positive BC (8,26). In the current study, coamplification of *CCND1* and *FGFR1* was observed in 4 amplified samples, which means that they possibly cooperate in oncogenesis. Coamplification of *FGFR1* and *C-MYC* occurred in 6 amplified samples, which might be either due to polyploidy of chromosome 8 or their synergistic roles. *FGFR1* amplification was also statistically correlated with tumor size, *C-MYC* status, and positive *ER* status.

Altogether, the applied qPCR strategy using generic dsDNA dyes in the present study revealed sensitive, accurate, and cost-effective abilities for determining the RCNs of the selected genes. Our findings also showed a considerable correlation between qPCR and FISH; however, a suitable cut-off point for qPCR is a prerequisite for determining the exact status of target genes. Microdissection is also proposed to take a pure cancer cell sample so as to eliminate normal cell contamination. The SYBR Green I assay could also be applied in a number of conditions where gene amplification is involved in disease etiology. Additionally, *CCND1*, *C-MYC*, and *FGFR1* amplifications seem to convey prognostic value. Coamplification of the selected genes proposes intergene cooperation and a synergistic role in BC tumorigenesis. Nonetheless, more studies with larger sample sizes are suggested to confirm our findings.

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