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Detection and evaluation of c-Myc oncoprotein in Egyptian patients with breast cancer

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Early breast cancer (BC) detection increases the possibility for successful and adequate disease treatment. There is no effective available BC biomarker, particularly in early-stage disease. We aimed to evaluate the association between c-Myc oncoprotein and BC development in Egyptian women. Also, to evaluate the association between its serum levels and some tumor severity features. A total of 172 females with BC (n=102), benign breast disease (n=40) and 30 healthy individuals constituted the present study. Data were collected from patient's histopathology reports. Serum c-Myc levels were detected by Western blot and enzyme linked immunosorbent assay (ELISA). Its diagnostic value was evaluated by ROC curve analysis. Single immunoreactive band corresponding to c-Myc oncoprotein (62 kDa) was observed in patients with BC than with benign diseases. BC patients were significantly (*P*<0.0001) associated with elevated c-Myc detection rate and level (78.4%; 1.7±0.03) compared to benign (35%; 1.2±0.02) or normal (10%; 0.2±0.01) groups. Moreover, these values increased with disease severity features including late stages (T4), high grades (grade III), lymph node involvement and positive distant organs metastasis. c-Myc discriminated BC patients from all non-cancer individuals with good diagnostive value (area under curve (AUC)=0.79; sensitivity=78.4%; specificity=75.7%, PPV=82.5%; NPV=70.7%; accuracy=77.3%). This good diagnostic rises when comparing BC patients to only healthy individuals. In conclusion, serum c-Myc levels can serve as promising biomarker to detect BC and thus may be used with other markers to overcome low sensitivity and thus may facilitate definitive treatments.

Keywords: Breast cancer, Diagnosis, c-Myc, Serum biomarkers, Disease severity.

INTRODUCTION

Among women, breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer death (Bray et al., 2018). It is the most common tumor among Egyptian women and constitutes 32% of National Cancer Institute cases (Ibrahim et al., 2014). Tumor early detection increases the possibility for successful and adequate disease treatment (Freitas et al., 2020).

BC diagnostic tools such as breast ultrasound or mammography may have the detection potential. However, they have limitations like radiation exposure and required BC size (at least a few millimeters) for detection (Attallah et al., 2014). Also, there is no effective blood-based marker available for BC detection, particularly in early-stage disease (Harris et al., 2007). For early diagnosis, cancer biomarkers detection in biological fluids is useful (Freitas et al., 2020). Moreover, to improve life quality of breast cancer

patients and disease outcomes, accurate and rapid determination of specific circulating biomarkers at different molecular levels with minimally or non- invasive methods constitutes a major challenge (Campuzano et al., 2017).

Since its discovery (Bishop, 1982), several studies demonstrated *c-myc* gene central role in proliferation and malignant transformation of animal and human cells (Liao and Dickson, 2000). It is a multifunctional oncogene that has been shown to be overexpressed and amplified in many types of human cancers, including BC, lung cancer, sarcoma, neuroblastoma, esophageal cancer and ovarian cancer (Dang, 2012). In several studies of ductal BCs, increased *c-myc* gene amplification was found in many cases (Deming et al., 2000). This amplification was associated with early relapse, high-grade BCs and poor prognosis (Chrzan et al., 2001; Blancato et al., 2004).

In this study, we aimed to evaluate the association between *c-myc* gene product (c-Myc, 62 KDa) and BC development in Egyptian women. On the other hand, we aimed to evaluate the association between its elevated circulating levels and some tumor severity features including late stages, high grades and positive lymph node and distant metastasis..

MATERIALS AND METHODS

Study participants and samples collection

A total of 172 participants recruited from Oncology Center, Mansoura hospitals, Mansoura, Egypt were as follow: female patients with BC (n=102) and others with benign breast disease (n=40) and a total of 30 healthy female individuals. Diagnosis of BC was determined by ultrasound, mammography, tomography or any combination of multiple modalities and was pathologically confirmed. All benign and healthy subjects had no personal BC or any cancer history. Data regarding age, tumor grade and stage, lymph node status and distant organ metastasis were collected from patient's histopathology reports. Before enrollment and sample collection, written informed consent was obtained from all participants; the study was implemented and designed in accordance with ethical guidelines in the Helsinki Declaration. Before starting any cancer-specific therapy, blood from all patients was drawn up to 3 days after diagnosis. Serum samples were decanted after blood centrifugation at 1,000×g for ten minutes and aliquoted and stored at -20°C until analysis.

Western blotting

According to Laemmli (1970), proteins of serum samples and molecular weight standards (Sigma, USA) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For assessing c-Myc expression, resolved proteins were electro-transferred onto 0.45 µm pore size nitrocellulose membrane (Sigma, USA) according to Towbin et al. (1979). After membrane blocking with Tris-buffered saline-Tween (pH 7.5) supplemented with 5% bovine serum albumin, it was probed with c-Myc monospecific antibody (ABC Diagnostics, New Damietta, Egypt) with constant shaking at 4°C overnight. Following washing, NC was incubated with conjugate (rabbit alkaline phosphatase IgG; Sigma, USA) for two hours. Antibodies dilutions were adjusted to eliminate target protein presence at low concentrations. Target protein was visualized by incubating NC with alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate [BCIP]/nitro-blue tetrazolium [NBT]) (Sigma, USA). Ten minutes later, the reaction stopped by distilled water and color development was observed.

Serum c-Myc detection using ELISA

As previously described (Attallah et al., 2017), circulating c-Myc was quantitively detected using ELISA. Samples were diluted in carbonate/bicarbonate buffer (pH 9.6) and 50 µL/each diluted sample allowed to coat onto ELISA plates overnight at 4°C (Costar, Corning Life Sciences, Acton, MA). Precoated plates were washed four times with phosphate buffered saline and non-specific sites were blocked using bovine serum albumin (0.2%) at room temperature. After four washes, the plates were incubated with of diluted monospecific antibody against c-Myc (50 µL/well) at 37 °C for two hours. After washing anti-rabbit IgG alkaline phosphatase conjugate (Sigma, USA) was added. After incubation and washing, the previously mention alkaline phosphatase substrate (Sigma, USA) was added for half hour at 37 °C. The reaction stopped with 3 M NaOH and the optical density was reading using microplate spectrophotometer (Metreiteck, Axiom, Burstadt, Germany) at 405 nm.

Statistical analysis

SPSS and GraphPad programs were used. Continuous variables were expressed as mean ± standard deviation (SD), whereas categorical variables were expressed as numbers or percentages. c-Myc positivity rates were compared among studied groups using chi square test. Other data were analyzed using ANOVA test and Fisher's Least Significant Difference was used as post hoc test. Significance level was determined at <0.05. Receiver operating-characteristic (ROC) curves were used for determining c-Myc diagnostic power. A 2 × 2 contingency table was used to drive common c-Myc performance indicators.

RESULTS

Circulating c-Myc is overexpressed in breast cancer

Data concerning females and tumor clinical findings are showed in Table 1. BC patients were older (*P* <0.01) than benign and healthy controls. Despite healthy women, single immunoreactive band corresponding to c-Myc oncoprotein (at 62 kDa) was observed in sera of patients with BC and benign diseases (Figure 1A). BC patients were associated with increased c-Myc positively rate as shown in Table 2. Interestingly, c-Myc detection rate was increase with disease severity features like, late tumor stages (T4) (95.2%), high grades (grade III) (92.9%), lymph node involvement (85.5%) and positive distant organs metastasis (90.9%) (Table 2).

Elevated c-Myc levels were associated with BC severity

Mean c-Myc absorbance levels (O.D) of BC patients (1.7±0.03) were significantly (*P*<0.0001) higher than that of benign (1.2±0.02) and healthy (0.2±0.01) groups (Figure 1B). Regarding histopathological features, elevated c-Myc serum levels were associated with late tumor stages (T3–T4), high tumor grades (Grade III), positive lymph node involvement and positive distant organs metastasis (Table 3).

Table 1: Clinicopathologic parameters of BC patients and age of studied groups

Variable	Value
Breast cancer patients (n=102)	
Age (mean±SD, years)	50.9±11.6
T stage, no. (%)	
T_1	21 (20.6)
T_2	35 (34.3)
T_3	25 (24.5)
T_4	21 (20.6)
Histological grade, no. (%)	
Grade I	15 (14.7)
Grade II	45 (44.1)
Grade III	42 (41.2)
Lymph node involvement, no. (%)	
Negative (N0)	33 (32.4)
Positive (N1)	69 (67.6)
Distant organs metastasis, no. (%)	
Negative (M0)	72 (70.6)
Positive (M1)	30 (29.4)
Patients with benign breast disease (n=40)	
Age (mean±SD, years)	46.4±11.2
Healthy females (n=30)	
Age (mean±SD, years)	45.6±11.3

Table 2: c-Myc detection rates among studied groups and according to different tumor features

Groups	c-Myc oncoprotein		X^2; *P* value
	Negative	Positive	
Control	27 (90%)	3 (10%)	42.8; <0.0001
Benign disorders	26 (65%)	14 (35%)	
Breast Cancer	22 (21.6%)	80 (78.4%)	
According to tumor stages			
T_1 (n=21)	9 (42.9%)	12 (57.1%)	10.8; *P* =0.013
T_2 (n=35)	9 (25.7%)	26 (74.3%)	
T_3 (n=25)	3 (12%)	22 (88%)	
T_4 (n=21)	1 (4.8%)	20 (95.2%)	
According to tumor grades			
Grade I (n=15)	7 (46.7%)	8 (53.3%)	11.4; 0.003
Grade II (n=45)	12 (26.7%)	33 (73.3%)	
Grade III (n=42)	3 (7.1%)	39 (92.9%)	
According to lymph node involvement			
Negative (n=33)	12 (36.4%)	21 (63.6%)	6.3; 0.012
Positive (n=69)	10 (14.5%)	59 (85.5%)	
According to distant organ metastasis			
Negative (n=72)	19 (26.4%)	53 (73.6%)	3.9; 0.046
Positive (n=30)	3 (10%)	27 (90%)	

Table 3: Level of c-Myc oncoprotein according to tumor severity features

Groups	c-Myc oncoprotein level (OD)	*P* value
According to tumor stages		
Early stage (T_1-T_2)	1.3 ± 0.01	<0.01
Late stage (T_3–T_4)	1.7 ± 0.02	
According to tumor grades		
Low grade (Grade I-II)	1.6±0.01	<0.05
High grade (Grade III)	1.9±0.01	
According to lymph node involvement		
Negative (N0)	1.4±0.01	<0.01
Positive (N1)	1.8±0.02	
According to distant organ metastasis		
Negative (M0)	1.5±0.02	<0.05
Positive (M1)	1.9±0.03	

Table 4: Diagnostic performance of c-Myc for differentiation BC patients from non-cancer individuals

Test results	Actual status		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)
	+	-					
Breast cancer vs. all non-cancer							
All non-cancer	17	53	78.4	75.7	82.5	70.7	77.3
Breast cancer	80	22					
Breast cancer vs. Healthy							
Healthy	3	27	78.4	90	96.4	55.1	81.1
Breast cancer	80	22					
Breast cancer vs. Benign							
Benign	14	26	78.4	65	85.1	54.2	74.6
Breast cancer	80	22					
PPV= Positive predictive value; NPV= Negative predictive value							

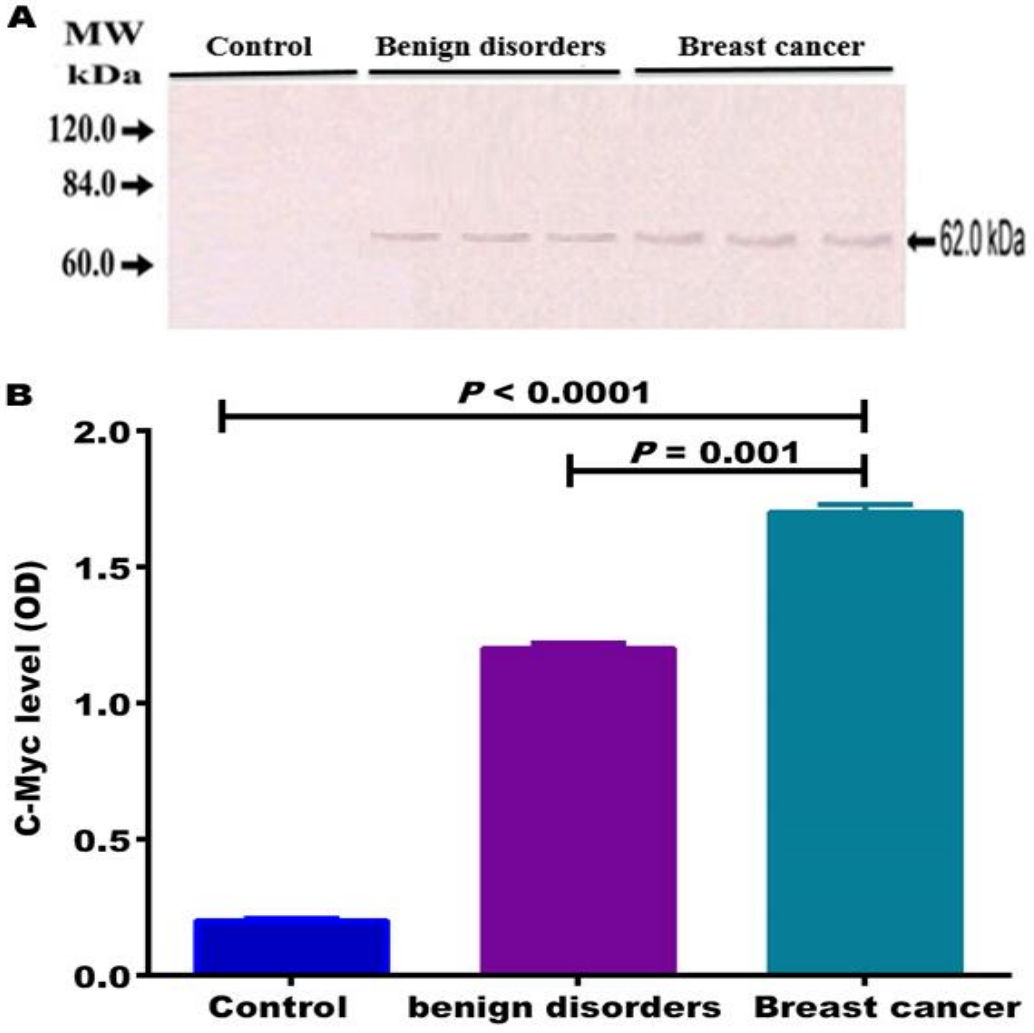

Figure 1. c-Myc is overexpressed in BC. (A) Expression of serum c-Myc at 62-kDa in healthy individuals and patients with benign diseases and BC detected by Western blotting. (B) Serum levels of c-Myc in BC was higher than non-cancer individuals (benign diseases and healthy controls) as measured by ELISA

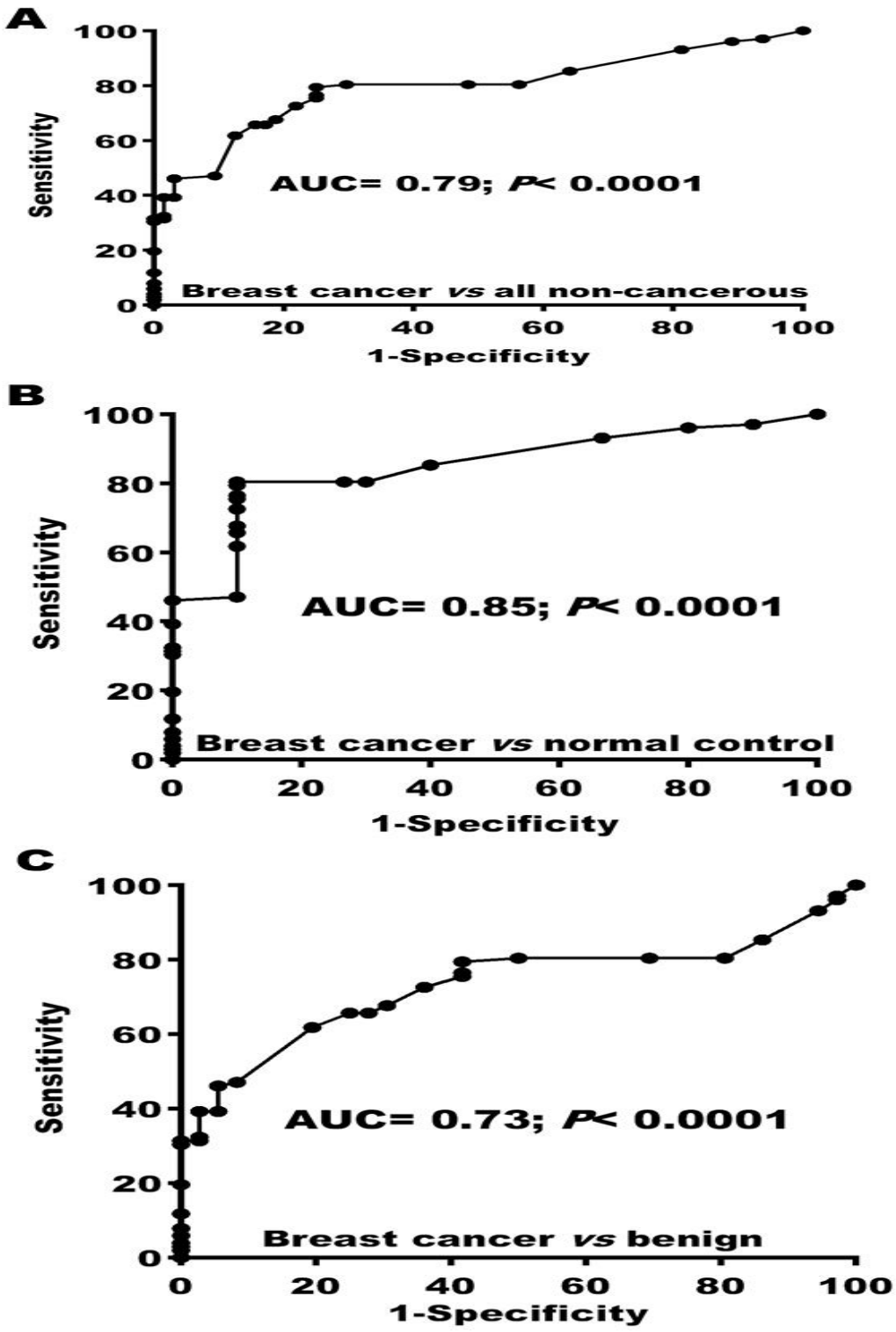

Figure 2: ROC analysis for c-Myc to discriminate BC patients from (A) all non-cancer individuals (normal and benign combined), (B) healthy individuals and (C) patients with benign breast diseases.

Diagnostic performances of c-Myc oncoprotein for identifying BC

Measurements of serum c-Myc were subjected to ROC curve analysis, and the results indicated that c-Myc can be used as BC biomarker capable of discriminating BC patients from all non-cancer individuals (AUC=0.79, *P*<0.0001; Figure 2A). This good diagnostic rises to 0.85 (*P*<0.0001) when comparing BC patients to only healthy individuals (Figure 2B). Moreover, it had good diagnostic power (AUC=0.73, *P*<0.0001; Figure 2C) when comparing BC patients to patients with benign diseases indicating its high diagnostic accuracy for BC. The calculated sensitivities, specificities, positive and negative predictive values and efficiencies were shown in Table 4.

DISCUSSION

This research aimed to evaluate c-Myc circulating levels in women with BC in comparison with patients with benign breast diseases and healthy controls. BC patients were older (*P* <0.01) than benign and healthy controls. Age have been reported to have a great impact on BC incidence. Most of diagnosed cases were over 40 years of age. In women <55 years of age, some studies reported that BC incidence appears to have a sigmoid function, with 1, 2.4 and 6.6% of all cases diagnosed before age 30, 35, 40, respectively (Anders et al., 2009).

Despite healthy controls, BC patients showed intense immunoreactive bands at 62-KDa compared to patients with benign diseases using c-Myc-specific antibodies and western blotting. *c-myc* gene product, 62 kDa oncoprotein, is mainly localized in cell nucleus and its expression levels are elevated in various types of tumors (Hilpert et al., 2001). In several studies using immunoblotting, anti-c-Myc antibodies specifically reacted, similar to our finding, with a 62 KDa protein (Ben-Mahrez et al., 1988; Attallah et al., 2017).

Using ELISA, there is a marked (*P*<0.0001) increase in positivity rate and levels of circulating c-Myc in BC patients (78.4%; 1.7±0.03) compared to benign (35%; 1.2±0.02) or normal (10%; 0.2±0.01) groups. There is strong evidence supported *c-myc* proto-oncogenes role in tumor development and progression. By multiple mechanisms, *c-myc* gene abnormal regulation can result in genomic instability, aberrant cell cycle control and phenotypic transformation (Deming et al., 2000). Interestingly, studies on transgenic mice and patient material have implicated *c-myc* gene overexpression in BC etiology (Sovak et al., 1997). In human primary BC, Bonilla et al. (1988) studied cellular *c-myc* proto-oncogene genomic organization. They found in most of BCs two types of alterations (rearrangement and amplification). In BCs with negative axillary nodes, group with high recurrence frequency, *c-myc* amplification showed significant relation with early and intermediate recurrence risks (Roux-Dosseto et al., 1992). *c-myc* gene amplification was reported to be independent powerful BC prognostic factor compared to HER2/neu amplification which may be have limited prognostic value (Berns et al., 1992). By immunohistochemistry, c-Myc protein was examined in normal breast tissues, non-invasive and invasive BCs. c-Myc was detected in some of normal breast tissues but in all BC specimens (Pavelic et al., 1991).

Indeed *c-myc* gene activation is associated with rapidly progressive and growing BC (Borg et al., 1992). Here, elevated c-Myc detection rates and serum levels were associated with late tumor stages (T3–T4), high tumor grades (Grade III), positive lymph node involvement and positive distant organs metastasis. Similarly, other studies reported that c-Myc amplification is the first identified genetic alteration that is related to BC progression from in situ to invasive BC stages (Robanus-Maandag et al., 2003; Corzo et al., 2006). Pavelic et al. (1991) reported that invasive BCs stained more frequently with c-Myc specific monoclonal antibody than non-invasive BCs. A more recent study by (Naab et al., 2018) also reported statistically significant associations between c-Myc amplification and BC Luminal B subtypes, BC stage, positive lymph node status and distant organ metastasis.

In this study, c-Myc have a good diagnostic power, as indicated by ROC analysis, for discriminating BC patients from all non-cancer individuals (AUC=0.79; sensitivity=78.4%; specificity=75.7%, PPV=82.5%; NPV=70.7%; accuracy=77.3%). This good diagnostic performance rises when comparing BC patients to only healthy individuals. Ismail et al. (2009), reported similar good c-Myc diagnostic power in BC detection (AUC=0.752). In a recent study, Shi et al. (2019) also reported similar diagnostic power of c-Myc for BC diagnosis with AUC =0.77, sensitivity =63.3% and specificity =81.8%.

CONCLUSION

Taken together, results of this study suggested that serum c-Myc levels may be used as BC

biomarker with good diagnostic value. This issue is attributed to its good sensitivity and specificity. However, further studies are needed to examine the prospective analysis of c-Myc with other established BC markers in larger multicentric studies.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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