ASSESSMENT OF *C-myc* ONCOGENE AMPLIFICATION IN BREAST CANCER

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Cancer evolves from the accumulation of mutations in the same cell and the deregulation of two classes of genes, oncogenes and tumor suppressor genes, the large majority of breast cancers arise in the epithelial cells of the breast (Vural *et al.*, 2014). MYC proteins are a family of transcription factors that lie at the nexus of chromatin, gene regulation, and cancer. It is estimated that more than 50% of all human malignancies display over expression of one *myc* family member, *C-myc* is the defining member of the family and is broadly over expressed in hematologic malignancies, as well as a wide spectrum of solid tumors (Lance and William, 2015). *MYC* gene amplification has been reported as a poor prognostic biomarker in 25% of breast tumors and is associated with tumor aggressiveness, including genetic instability, high tumor grade, and estrogen receptor negativity (Grushko *et al.*, 2004). Polymerase chain reaction (PCR) is used for examination the state of amplification of the proto-oncogene *C-myc* in archival breast and ovarian carcinomas (Schreiber and Dubeau, 1990).

The present study aimed to assessment of *C-myc* oncogene amplification in 50 cases of invasive duct carcinoma using PCR technique and Correlation between *C-myc* amplification with estrogen; progesterone receptors and the human epidermal growth factor receptor-2 (HER2) was performed.

MATERIALS AND METHODS

Formalin fixed paraffin embedded tissues (FFPE) from 50 cases of infiltrating breast cancers were obtained from the archives of the Department of Pathology, National Cancer Institute, Cairo University. From each block 4 sections (5 µm thick) were obtained for Hematoxylin and Eosin staining (H&E) and estrogen; progesterone receptor and HER2 by immunostaining.

The histopathological diagnosis of all cases was revised by examining H&E

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stained slides (Bancroft and Gamble, 2002).

Immunostaining

Paraffin sections were processed after a standard procedure including blocking endogenous peroxidase activity in 1.0% hydrogen peroxide in PBS for 15 min. and antigen retrieval performed by microwave heating in citrate buffer, pH 6. The monoclonal antibodies, Excess serum was shacked off without washing and the slides were dried around edge of the section. Enough primary antibodies for ER (DAKO Cytomation, clone1D5, diloted at 1:80) and PR (DAKO Cytomation, clone636, diloted at 1:100) were added to each section and incubation a humid chamber at room temperature for 2 hour was carried out. Streptavidin-biotin immunoperoxidase method was used for each section (Dako, Universal LSAB_2 kit). 3, 3` diaminobenzidine tetrahydrochloride (DAB) solution was used as the final chromogen, and sections were counterstained with Mayer's hematoxylin before mounting. Negative controls for non-specific binding; incubated with secondary antibodies only; were processed and revealed no signals. Positive controls recommended by manufacturer were used to confirm correct immunohistochemical staining.

Immuostaining interpretation

Scoring immunostaining results was performed for nuclear staining for ER and PR according to Allred *et al*. (1998), while scoring for Her 2 neu membranous staining for overexpression was performed according to Wolff *et al*. (2007).

C myc molecular methods

DNA extraction

DNA extraction from paraffin sections was carried out according to Coombs *et al*. (1999). According to Maniatis *et al*. (1989), the concentration and purity of DNA were assessed by measuring light absorbency by DNA at 260 and 280 nm wave lengths in a spectrophotometer. The concentration was calculated using the formula:

DNA concentration = A260 × dilution factor 50 × 0.001 µg/µl.

The protein content in extracted DNA was measured by calculating the ratio A260 to A280. Ratio greater than 1.7 was considered optimum.

Polymerase chain reaction (PCR) amplification

Gene copy determination using polymerase chain reaction was done according to (Lőnn *et al.*, 1995).

Samples containing 0.25 µg of genomic DNA were amplified for 20 cycles in a thermal cycler, after an initial denaturation step at 95°C for 5 minutes, in a 100 µl final volume containing 20 nmol/L of oligonucleotide primers, each of (c-myc/thymidine kinase), 200 nmol/L each of deoxyadenosine triphosphate (dATP) (20 µl), deoxycystidine triphosphate

(dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP) (Sigma), 1.5 U Taq polymerase (Boehringer Mannheim), and PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, 2.25 mmol/L Mg Cl_2 and 0.01% gelatin, Boehringer Mannheim). Primers were obtained from Gibco BRL, USA. The C-myc and thymidine kinase primers sequences used are presented in Table (1). PCR was performed in the thermal cycler, RoobyCycler gradient 96 Stratagene. Samples were heated for 5 min at 94°C for denaturation, and then 20 cycles at 94°C for 1 min, 56°C for 2 min, and 72°C for 3 min, followed by a final extension cycle at 72°C for 5 min.

Analysis of PCR products

Electrophoretic separation of PCR products was conducted on 2% agarose gel. 10 ul of PCR product was mixed with 2 µl of 6x loading dye, and then loaded into the wells of the agarose gel stained with ethidium bromide. Samples were separated under constant voltage (80V) for 30 min, DNA molecular weight marker Qiagen Gelpilot® 50 was used, DNA was visualized by exposure the gel to UV transilluminator.

Assessment of C-myc gene amplification

Semiquantitation of *C-myc* gene by co-amplification of c-myc gene and thymidine kinase as a control gene was assessed. Gel was analyzed by a documentation system, using phoenix 1D software V 5.1, to measure the density of each amplified fragment.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 software (Chicago, USA). Results are expressed as mean ± standard deviation (SD). Correlation is considered significant when $p \leq 0.05$ (Saunders and Trapp, 2001).

RESULTS AND DISCUSSION

Breast carcinoma is an important cause of mobility and mortality among women, carcinoma of the breast is the most prevalent cancer among Egyptian women and constitutes 33% of National Cancer Institute cases (El-Bolkainy *et al*., 2013). This research was performed to assess *C-myc* oncogene amplification by semi-quantitative PCR approach in breast cancers and results were correlated to biological indicators to established prognostic factors as well as estrogen , progesterone receptors status and *HER2* oncogene using immunohistochemical staining. Invasive ductal breast cancer is the most common type of breast cancer accounting for about 75% of all invasive breast cancers. It affects the ducts and lobules of the breast and had the potential to spread widely. The normal breast tissues are composed of 15-20 sections, called lobes which end among lobules. These lobules further end in tiny bulbs which produce milk during lactation (Fig. 1 A). Malignant breast tissues invasive duct carcinoma showed groups and clusters of malignant ductal cells, of highly anaplasia and mitosis (Fig. 1 B). In the present study out of 50 IDC, 30 (60%) tumors showed positive nuclear

immunostaining for ER, while 20 (40%) were negative (Table 2), (Fig. 2 A and B). Among 50 breast cancer cases studied nuclear positivity for progesterone receptors (PR) was detected in 40%, while 60% of tumors were negative for progesterone receptors (Table 2), (Fig. 2 C and D). Immuno-histochemical detection of *HER2* gene revealed positivity (score 2, 3) in 16 cases (32%), and were negative (score 0 and 1) in 34 cases (68%) (Table 2 and Fig. 2 E and F). None of normal breast tissue samples revealed *C-myc* gene amplification. Twenty two cases (44%) showed *C-myc* oncogene amplification (molar ratio of *C-myc* gene to reference gene TK >1; range from 1.01-1.7), while 28 cases were negative for *C-myc* oncogene amplification (Fig. 3 A and B). Previous studies showed an average of 15.5%, of breast cancer biopsies bear *C-myc* gene amplification of three fold or greater (Liao and Dickson, 2000). Lőnn *et al*. (1995) found that *C-myc* amplification was present in 16% of the cases. Another report revealed that 22% of the tumor cases showed increased *C-myc* mRNA expression, and the over-expression was rarely due to the gene amplification (Bieche *et al*., 1999). Table (3) shows inverse statistically significant association between *C-myc* and estrogen receptors; 31.8% (7/22) of *C-myc* positive cases were ER positive, While 67.9% (19/28) of negative *C-myc* gene cases were ER positive. Inverse statistically significant association was detected between *C-myc* gene and progesterone receptors (PR) expression by immunohistochemistry; 22.7% (5/22) of *C-myc* gene positive cases were PR positive, while 71.4% (20/28) of negative *C-myc* gene cases were PR positive. Direct statistically significant association was detected between *C-myc* gene amplification by PCR and HER2 expression by immunohistochemistry; 72.7% (16/22) of *C-myc* gene positive cases were HER2 positive, while 64.3% (18/28) of negative *C-myc* cases were HER2 positive. In breast cancer, amplification of *C-myc* may correlate positively or negatively with alterations in other genes. Studies showed that amplification or overexpression of *C-myc* occurs more frequently in the cases that are negative for estrogen receptor (ER-) (Persons *et al*., 1997; Bolufer *et al*., 1994) and/or progesterone receptor (PR-) (Berns *et al.*, 1992). Overexpression or amplification of the *C-myc* gene has been observed to occur preferentially in PR-negative breast cancer cases (Berns *et al*., 1992). Another study reported that there was no relationship between the amplification of *HER2* or *myc* and ER, PR or tumor size (Lőnn *et al*., 1995).

Therefore, it is evident from the aforementioned results that *C-myc* amplification is associated with unfavourable breast carcinoma with her 2 neu overexpression and negative ER and PR expression. Amplification of *C-myc* gene show prognostic value in patients with operable breast cancer. The results show that Formalin fixed breast tumors material can be successfully used for DNA/PCR analysis, and it is very suitable for determining the presence/absence of gene amplification to

obtain prognostic information. Finally it could be concluded that *C-myc* could be considered as prognostic markers in cancer breast. Routine immunostaining of *C-myc* for all cases of breast cancer, in addition to ER, PR and Her-2.

SUMMARY

Breast carcinoma is an important cause of mobility and mortality among women. Carcinoma of the breast is the most prevalent cancer among Egyptian women and constitutes 33% of National Cancer Institute cases. The present study aimed to assessment of *C-myc* oncogene amplification in 50 cases of invasive duct carcinoma using PCR technique and correlation between *C-myc* amplification with estrogen; progesterone receptors and human epidermal growth factor receptor-2 (HER2) was performed. Formalin fixed paraffin embedded tissues (FFPE) from 50 cases of infiltrating breast cancers were obtained from the archives of the department of Pathology, National Cancer Institute, Cairo University. Thirty tumors (60%) showed positive nuclear immunostaining for ER, while 20 (40%) were negative. Immunohistochemical detection of *HER2* gene revealed positivity (score 2, 3) in 16 cases (32%). Twenty two cases (44%) showed c-myc oncogene amplification (molar ratio of *C-myc* gene to reference gene TK>1; range from 1.01-1.7. Therefore, it is evident from the aforementioned results that *C-myc* amplification is associated with unfavourable breast carcinoma with HER 2 neu overexpression and negative ER and PR expression. Amplification of *C-myc* gene show prognostic value in patients with operable breast cancer. The results show that Formalin fixed breast tumors material can be successfully used for DNA/PCR analysis, and it is very suitable for determining the presence/absence of gene amplification to obtain prognostic information. Finally it could be concluded that *C-myc* could be considered as prognostic markers in cancer breast. Routine Immunostaining of *C-myc* for all cases of breast cancer, in addition to ER, PR and Her-2.

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Table (1): Primers used to amplify *C-myc* gene and thymidine kinase.

Primer name	Nucleotide sequence	Expected size (bp)
C-myc-P1	5`-GTTTCATCGTGTTGGCCAGGATGGT-3`	119
C-myc-p2	5`-CCAAAGAGCCACTCTAAGCCTGGT-3`	
TK-P1	5`-CTCTGGGAACAACTCTGGGATGAGG-3`	136
TK-P2	5`-ACTCAGGTGGTCCCAGGAAGTGTGG-3`	

Table (2): Immunohistochemical data in breast cancer patients

Immunohistochemical parameters		No. of cases	%
c-erbB-2	+ve	16	32
	-ve	34	68
ER status	+ve	30	60
	-ve	20	40
PR status	+ve	20	40
	-ve	30	60

Table (3): Correlation between *C-myc* gene amplification by PCR and some molecular prognostic markers.

Molecular prognostic markers		*C-myc* gene amplification				P. Value
		+ve		-ve		
		No. of cases (22)	%	No. of cases (28)	%	
ER	+ve	7	31.8	19	67.9	(p<0.0001) ***
	-ve	15	68.2	9	32.1	
PR	+ve	5	22.7	20	71.4	(p<0.0001) ***
	-ve	17	77.3	8	28.6	
Cerb-2	+ve	16	72.7	18	64.3	(p<0.0001) ***
	-ve	6	27.3	10	35.7	

*: Mild significance; **: Moderate significance; ***: Highly significant.

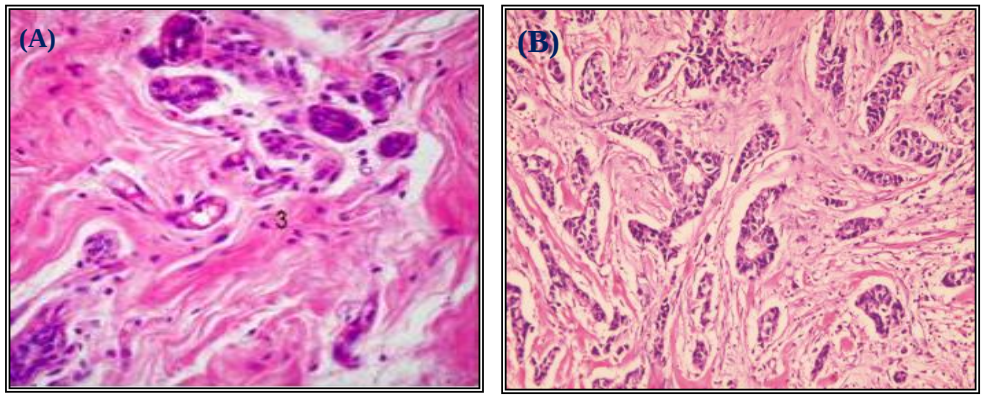

Fig. (1): Histological studies. Where: (A) Normal breast lobules by H and E (x 400), (B) Invasive duct carcinoma, H and E (x 400).

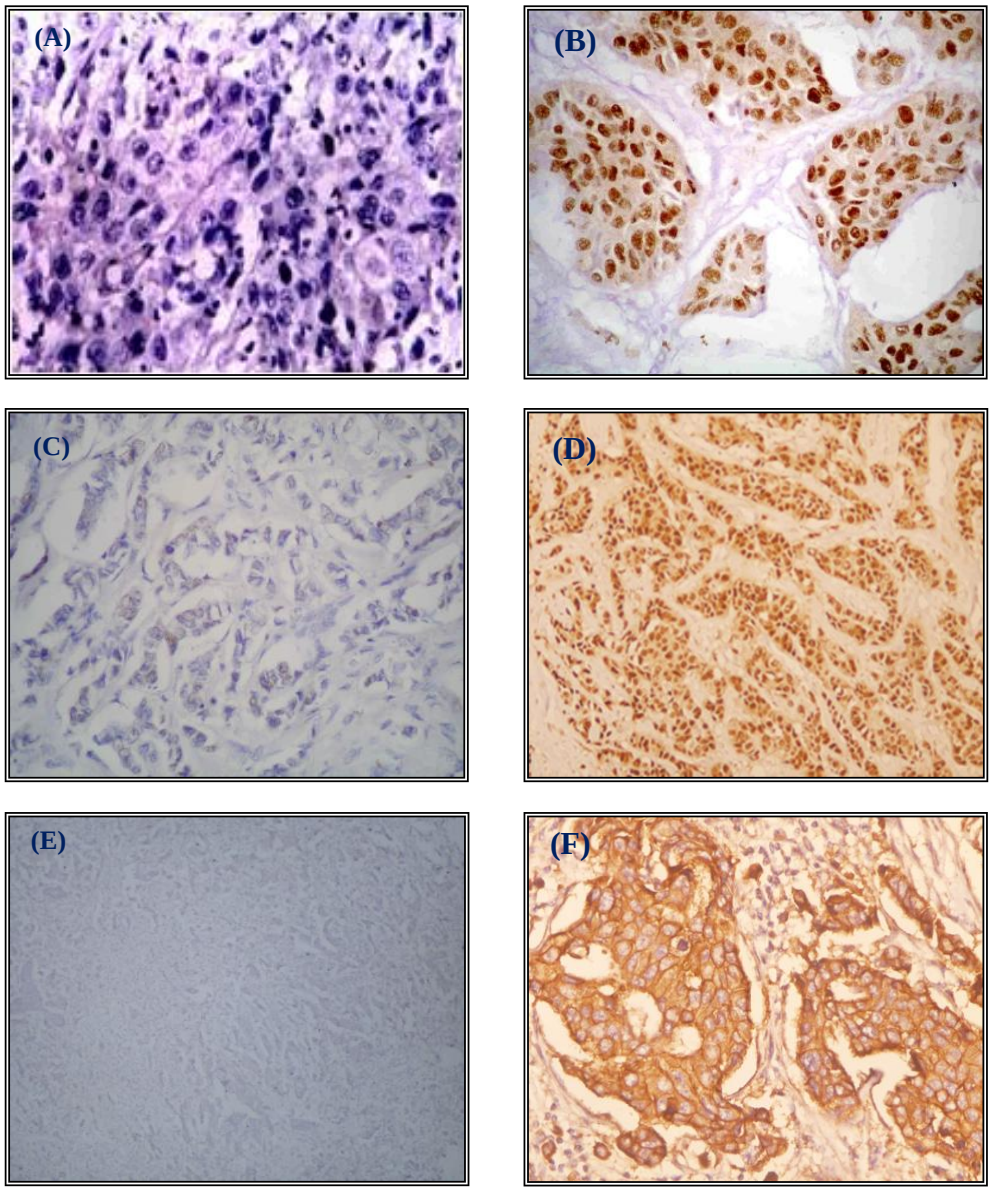

Fig. (2): Immuohistochestry for ER, PR receptors and *HER-2* gene. (A) Invasive duct carcinoma showing negative for ER x400, (B) Invasive duct carcinoma showing positive for ER x400, (C) Invasive duct carcinoma showing negative for PR x400, (D) A Case of cancer breast with IDC showing positive for PR X400, (E) Invasive duct carcinoma showing negative for HER -2 expression (x400) and (F) Invasive duct carcinoma showing positive for HER2 expression (x400).

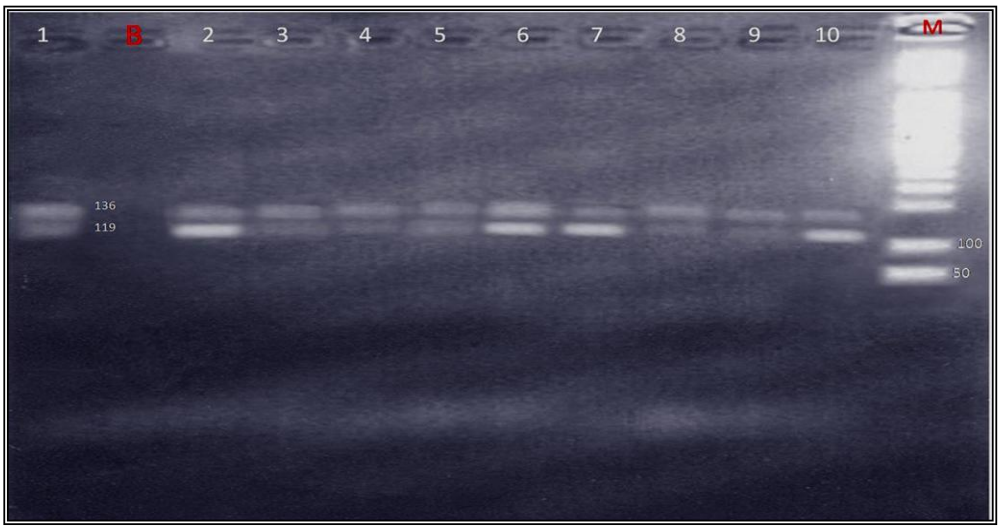

Fig. (3 A): Detection of *C-myc* gene and its amplification in IDC. Electrophoresis separation of *C-myc* gene (119 bp) and control gene *thymidine kinase* (136 bp), PCR amplified fragments on 2% agarose gel. Lane 1: negative normal breast tissues as a control. Lane B: Blank as a control. Lanes 3, 4, 5, 8 and 9: IDC negative and lanes: 2, 6, 7 and 10 positive for *C-myc* gene amplification. Lane M: 50 bp molecular weight DNA standard.

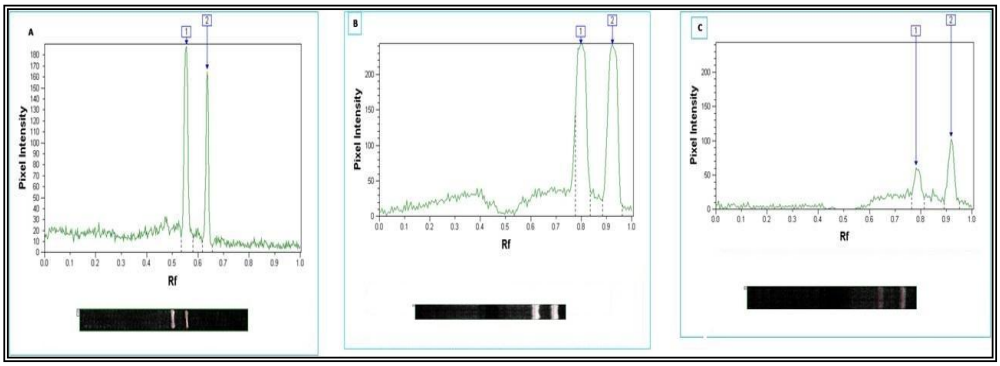

Fig. (3 B): Electrophoretic pattern of PCR products from (A) negative normal breast tissues controls showed a molar ratio of *C-myc* /*TK* < 1 (1- *Thymidine Kinase* and 2- *C-myc* gene); (B) Invasive duct carcinoma: negative revealed a molar ratio of *C-myc* /*TK* <1 (2-10 copies) and (C) Invasive duct carcinoma: positive revealed a molar ratio of *C-myc* / *TK* >1 (2-10 copies).