World Journal of Medical Sciences 13 (2): 151-156, 2016
ISSN 1817-3055

DOI: 10.5829/idosi.wjms.2016.13.2.1162

Evaluation of Estrogen and Progesterone Receptors, Together with Ki-67 and p27 in Breast Invasive Duct Carcinoma

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Abstract: p27 is a negative regulator of cell cycle, considered a tumor suppressor gene and belongs to a family of cyclin-dependent protein kinase inhibitors. The aim of this work is to study the value and importance of p27 expression as a potential prognostic marker in breast invasive duct carcinomas (NST).The examined cases were immunostained by p27 and correlated with established prognostic factors of breast carcinoma (tumor stage, grade, lymph node status, ER, PR, Ki-67 index). The recorded results show statistically significant relationship between diminished p27 expression and aggressive parameters of the examined tumors (you should mention them). It can be concluded that p27 is a potential prognostic and predictive marker and may play a role in designing an individually tailored cancer therapy.

Key words: Breast invasive duct carcinoma · Cell cycle · p27 · Prognosis

INTRODUCTION

Breast cancer is the most common carcinoma in women accounting for 23% of all cancers worldwide and 27% in developed countries [1]. A study upon Egyptian patients reported that the commonest sites of cancer in females are breast (38.8%) [2]. Recent gene expression studies have confirmed that breast cancer is no longer a single disease with variable morphology, but diverse types with high molecular heterogeneity [3]. Since then certain immunohistochemical markers can provide prognosis and predictive treatment information and also have easy clinical accessibility [4]. These have been used in an attempt to determine intrinsic subtypes. A simplified immunohistochemical classification including Estrogen receptor (ER), Progesterone receptor (PR), Her 2/neu and Ki-67 index is now considered a surrogate for establishing breast cancer subtype [5]. p27 and skp2 are negative regulators of the cell cycle are considered tumour suppressor genes that a loss of their function can contribute to malignant behaviour [6]. In breast cancer, diminished expression of p27 is associated with shorter overall survival and shorter time to progression and this seems to be a stronger independent predictor of outcome than either p53 alterations or tumor grade [7]. Quiescent normal epithelial cells in most body organs express high levels of nuclear p27. This is reduced in premalignant and non-invasive cancerous lesions, including mammary ductal carcinoma in situ [8]. Therefore, the present study aims to Estimation of p27 expression in breast invasive duct carcinoma (NST) and correlation between p27 expression with established prognostic factors of the breast cancer (Tumor stage, grade, lymph node status, ER, PR, Ki-67 index).

MATERIALS AND METHODS

Study Group: This is a retrospective study including retrieval of formalin fixed paraffin embedded tissue sections from archival blocks of forty female cases of breast invasive duct carcinoma (NST), collected from Egyptian patients from different labs from period of January 2013 to January 2015. Cases underwent mastectomy for proper evaluation of axillary lymph nodes. Patient and tumor characteristics were retrieved from clinical records and pathology reports.

Histopathological Evaluation: conducted on H and E stained sections from paraffin blocks. Histologic grading of invasive duct carcinoma was done according to Elston-Ellis modification of Bloom-Richardson grading system (Nottingham grading system) [9]. Tumor staging was estimated according to latest WHO staging system (AJCC) (American Joint Committee on Cancer) [10].

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Immunohistochemical Procedure: Sections of 4 microns in thickness were deparaffinized in xylene, then were hydrated through a series of graded alcohols (95%-70%), distilled water and phosphate buffered saline (at pH 7.5). The slides were then immersed in 10 mm citrate buffer (pH 6) and were twice pretreated by microwaving oven 800W for 4 then 8 minutes. Between each period of heating, evaporated fluid was replenished. After a 25 minute cooling period the endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide (H_2O_2) for 5 minutes. Antigen retrieval was done by immersing the slides in 10 mm citrate buffer (pH 6) for 10-20 minutes at 100°C in a microwave followed by cooling at room temperature for 20 minutes. The tissueswere blocked with protein blocking reagent for 30 minutes to reduce nonspecific staining. After washing with Tris-buffered saline sections were incubated with the primary antibody for 1 hour at room temperature (for ER, PR, and Ki-67 all of them are monoclonal antibodies, Dako, ready to use) (p27 is a monoclonal antibody, Novus, ready to use) The sections were washed in Tris-buffer abd incubated with avidin-biotin-peroxidase system (DAKO) for 30 minutes. The excess reagent was tapped off and the slides were washed with PBS (phosphate buffer solution) and dried. All slides were rinsed well in tap water for 5 minutes then slightly counterstained with Mayer's hematoxylin for 1-2 minutes. The slides were cleared in xylene for 3 changes and then Canada balsam and cover slips were applied. Cases were considered to stain positively if all or at least clusters of tumor cells display a brown color. Positive controls were considered: (p27 has internal positive control as lymphocytes and normal breast ducts) (ER and PR positive control is normal breast ducts) (Ki-67 positive control is tonsil).

Interpretation of Results and Statistical Analysis: ER and PR scoring was evaluated according to ASCO\CAP scoring recommendations guidelines [11]. Most prognostic analysis have scored p27 based on the percentage of tumor nuclei positive expression, using various cut-offs ranging from 0-50% with the most significant correlation obtained when comparing p27 score at 50% level [8]. Various cut off values for Ki-67 in breast cancer were considered following the correlation analysis for the Ki-67 index and other prognostic factors, a Ki-67 value ≥15% was revealed to be the optimal cut-off level for breast cancer patients [12]. Data was statistically described in terms of range, mean, median, frequencies (number of cases) and percentages when appropriate. SPSS (statistical package for social science) program, comparison of quantitative variables between the study groups was done using Mann Whitney U test for independent samples. Chi Square (X^2) test was performed. Correlation between the different study variables was done using Spearman correlation equation. A probability value (P value) less than 0.005 was considered statistically significant [13].

RESULTS

All the examined cases were invasive duct carcinoma (NST). Regarding tumor grade, thirty four cases were grade II (85%), five cases were grade III (12.5%) and one case was grade I (2.5%). Tumor size in this study ranged from 0.5 to 6 cm, with a mean size 3.125 cm (±). Cases were categorized according to AJCC pathologic staging 7th edition 2009. PT (primary tumors) was divided into three groups. The majority of cases in this study were PT2 (32) = 80%, PT1 (6) =15%. PT3 (2) = 5% of total cases. None of the examined cases was PTx, PT0 or PT4. Twenty five samples (62.5%) out of the studied forty cases showed positive axillary lymph nodes metastatic tumor deposits with the following further distribution:N1 (1-3 Lns)-14 cases -35%, N2 (4-9 Lns)- 8 cases-20% and N3 (10 or more L ns)-3 cases-7.5%. Steroid hormone receptor status was evaluated according to ASCO scoring guidelines and recommendations (2010) among the studied sample, thirty two cases (80%) showed positive staining for both ER (Fig 1) and PR (Fig. 2) receptors. Seven cases (17.5%) showed negative reactions for both ER and PR receptors. One case (2.5%) was positive for ER but negative for PR.None of the examined cases was ER negative, PR positive.

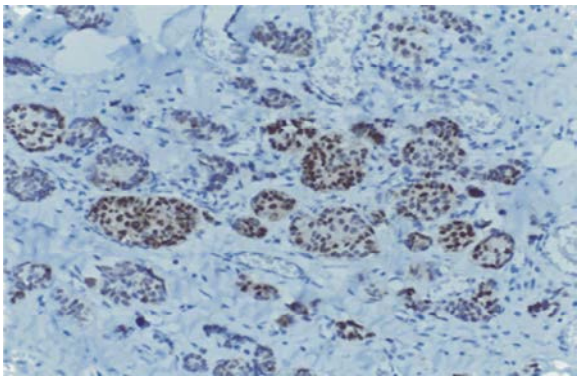

Fig. 1: A case of invasive duct carcinoma (NST) immunostained positive for ER x 400.

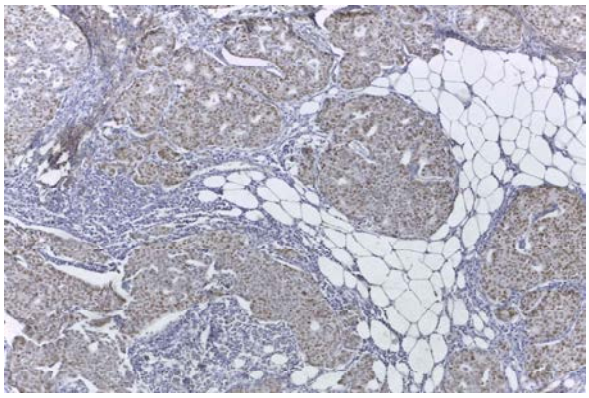

Fig. 2: A case of invasive duct carcinoma (NST) positive for PR x 400.

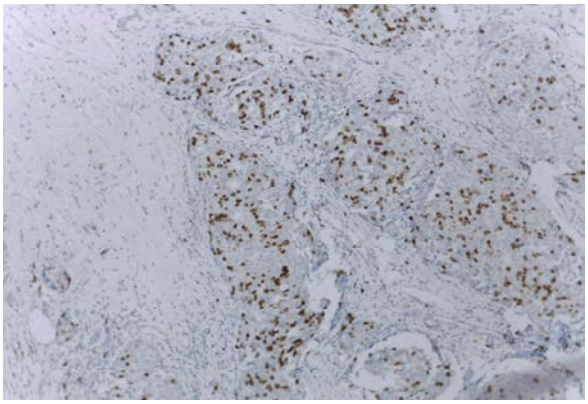

Fig. 3: A case of invasive duct carcinoma (NST), immunostained by Ki-67x 200, Ki-67 index 30%.

In this study 15% level is taken as a convenient cut off point for ki-67 scoring, so that those tumors expressing less than or equal to 15% positive nuclear immunostaining for Ki-67 are considered low expression (n=23) (57.5%), while those expressing more than 15% are considered high expression (n=17) (42.5%) (Fig 3). Ki-67 scoring in the studied sample ranged from 3 to 70 % with median value 22.5%. None of the examined cases was negative for p27. p27 scoring in this study ranged from 10% up to 90% with median percentage at 62.5%, mean value 62%. In this study 50% level is taken as a convenient cut-off point for p27 scoring, so that tumors recording less than or equal to 50% positive cells for p27 are considered low expressers (8 cases =20%), while those expressing more than 50% are considered high expressers (32 cases = 80%) (Fig 4). Grade I case highly expresses P27 (100%) and 30 out of 34 grade II cases (88.2%) highly express p27 and one out of five grade III cases highly express P27 (20%) (p value =0.003). In the examined samples

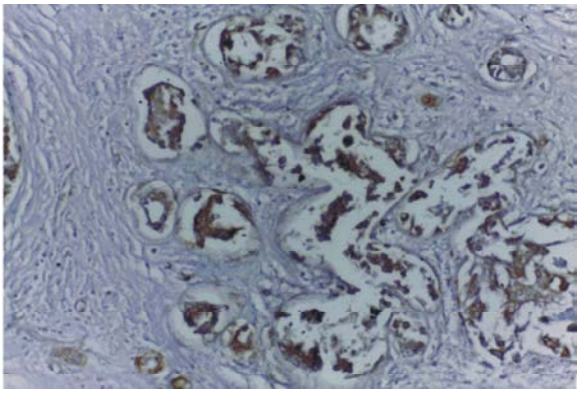

Fig. 4: A case of invasive duct carcinoma (NST), immunostained by p27 x400, score 80%.

all T1 tumors highly express p27, while 84.37% of T2 highly express p27 and all T3 tumors highly express p27 (p value =1).Among 15 studied cases without evident axillary lymph nodes metastatic deposits, 13 cases highly express p27 (86.6%). Among N1 studied 14 cases, 13 cases highly express p27 (92.8%). Among 8 studied N2 cases 4 cases highly express p27 (50%). Among 3 studied N3 cases, two of them highly express p27 (66.6%) (p value =0.013). Among the studied ER positive 33 cases, 28 of them are high expressors >50% for p27 (84.4%). Among the ER negative 7 cases, one of them only highly expresses p27 (14.28%). (P value =<0.001). Among the studied 32 PR positive cases, 29 of them highly express p27(90.6%), while among 8 PR negative cases, only 2 cases highly express P27(25%) (P value = <0.001). Among the studied 23 cases which express Ki-67 ≤15%, 21 of them highly express p27 (91.3%) while the 17 cases with high Ki-67 index (Ki-67 >15%) 11 of them highly express p27 (64.7%) (P value=0.043) (Table 1).

Table 1: Relation between p27 expression and tumor parameters.

Item	P27 high expressor>50%	P27 low expressor =50%
Grade I, II	88.57%	11.42%
Grade III	20%	80%
Stage pT1	100%	0%
Stage pT2-T3	85.3%	14.7%
N0-N1	89.6%	10.4%
N2-N3	54.5%	45.5%
ER positive	84.8%	15.2%
ER negative	14.28%	85.2%
PR positive	90.6%	9.4%
PR negative	25%	75%
Ki-67 low expresser	91.3%	8.7%
Ki-67 high expresser	64.7%	35.3%

DISCUSSION

Breast cancer is the most common carcinoma in women accounting for 23% of all cancers worldwide and 27% in developed countries [1]. Recent gene expression studies have confirmed that breast cancer is no longer a single disease with variable morphology, but with high molecular heterogeneity [3]. Since certain immunohistochemical (IHC) markers can provide prognosis and predictive treatment information and also have easy clinical accessibility [4]. These studyincluded forty cases of breast invasive duct carcinoma (NST) collected from Egyptian female patients. All the examined cases were evaluated immunohistochemically as regards ER, PR, Ki67 and p27 expression. Regarding tumor grade, thirty four cases were grade II (85%), five cases were grade III (12.5%) and only one case was grade I (2.5%). Our results are relatively close to what reported by Mokhtar *et al.* [14] in a study upon Egyptian patients and revealed grade I tumors (0.88%), grade II tumors (84%), grade III tumors (15.12%) and this may be attributed to later presentation of breast cancer among Egyptian patients together with genetic and geographic variability. The tumor size in this study ranged from 0.5 to 6 cm with the mean size 3.125 cm. P (T) primary tumor divided into three groups. The majority of cases in this study were pT2 (n=32) =80%, pT1 (n=6) =15%, pT3 (n=2) =5%. None of the examined cases was pTx, pT0, or pT4. These results contradicted with those reported by Barbareschi *et al.* [15], higher incidence of T1 breast carcinomas (43.5%) was detected. The differences may exist because most of Egyptian patients lack investigations for early detection of breast carcinoma. However results in this research are less advanced than those reported by El-Bolkainy *et al.* [16] where clinical (T) categories in NCI series were as follows (T1) (1.2%), (T2) (30%), (T3) (26.4%) thus indicating the improvement in screening methods and the better public awareness of the importance of early detection of breast cancer among Egyptian population.

Regarding metastatic tumor deposits to regional lymph nodes: In this study twenty five cases (62.5%) out of the studied forty cases showed positive axillary lymph nodes metastatic deposits with the following distribution: N1; 14 cases (35%), N2: 8 cases (20%), N3: 3 cases (7.5%).%. This is comparable with what reported by Nouh *et al.* [17] that the frequency of lymph node metastases is 40-50 % in western series, but a higher figure 70.6% reported in Egyptian patients. ASCO\CAP Elizabeth *et al.* [11] recommended reporting hormone receptor status in breast cancer as either positive or negative. In this study 80% (32 cases) of the studied population showed positive staining for both ER and PR receptors. Seven cases (17.5%) showed negative reaction for both ER and PR. Only one case (2.5%) was positive for ER but negative for PR. Our results are close to what reported by Ren *et al.* [18], in a study carried out on 439,444 patients with breast cancer revealed that 77.5% had ER positive disease. Also our results are comparable with what reported by Faheem *et al.* [19] in a study carried upon 1226 patients and revealed ER, PR and Her2/neu positive in 763 (62.2%), 738 (60.1%) and 478 (38.9%) patients, respectively.

Regarding Ki-67 index, the Ki-67 index ranged from 3 to 70 % with median value 25%, mean value 18%. This is comparable with what reported by Haroon *et al.* [20], they examined 194 cases of newly diagnosed breast cancer, Ki-67 index ranged from 1 to 90 % with mean 26.9%.

In the last few years, interest has grown in understanding the role and implication of p27 levels in breast cancer as a new valuable tool in prediction of prognosis in women with breast cancer. In this study none of the examined cases was negative for p27, i.e., all cases have expressed positive nuclear staining for p27 and variable degree of cytoplasmic staining. The percentages of nuclear expression among cases ranged from 10% up to 90% with median percentage among all the studied cases 62.5%. This is in agreement with those obtained by Chu *et al.* [8], who explained why p27 is rarely entirely lost in human malignancies by being incorporated in the regulation of cell motility, cell migration, invasion and metastasis. In this study 50% level is taken as a convenient cut-off, so that those tumors recording less than or equal to 50% positive cells for p27 are considered low expressors (8 cases =20%), while those expressing more than 50% are considered high expressers (32 cases =80%).

As Regards Tumor Grade: Grade I case highly express p27 (100%), 30 out of 34(88%) grade II cases highly express p27 and one out of five (20%) grade III cases highly express p27 denoting a significant relationship (p value =0.003) between tumor grade and p27 expression. This is close to results obtained by Fredersdorf *et al.* [21] as they found high level of p27 nuclear expression in 92% of grade I breast duct carcinoma, 80.5% of grade II tumors, only 30% of grade III duct carcinomas highly express p27. As regards tumor size (stage) p27 is highly expressed in 100% of T1 tumors. 85.3% of T2 and T3 tumors were high

expressers for p27 while 14.7% were low expressers for p27. Correlation revealed a non-significant relationship (p value =1.00). For lymph node status, among 15 studied cases without evident axillary lymph nodes metastatic deposits, 13 cases highly express p27 (86.9%), among N1studied 14 cases, 13 cases highly express p27 (92.8%), among 8 studied N2 cases 4 cases highly express p27 (50%), among 3 studied N3 cases 2 of them highly express p27 (66.6%). These results show a significant relationship (P value 0.013) between p27 expression and nodal status. This goes with what observed by Anderson *et al*. [22] that decreased p27 protein expression strongly correlates with lymph node metastases (P value <0.0001).

Considering hormone receptor status, among the studied ER positive 33 cases, 28 of them highly express p27 (84.8%); Among ER negative 7 cases, only one case highly express p27(14.2%), denoting a highly significant relationship between p27 expression and ER status (P value <0.001); Among studied 32 PR positive cases 29(90.6%) of them highly express p27 while among 8 PR negative cases, only 2 (25%) cases highly express p27 with a detected significant relationship (P value <0.001). These results came in concordance with results obtained by Catzavelos *et al.* [23],a group of the first researchers studying the prognostic role of p27 in breast cancer, demonstrated high significance relation for p27 with both ER and PR (P value 0.001 for each). Concerning of Ki-67, among the studied 23 cases with Ki-67 ≤15%, 21 (91.3%) of them highly express p27, while the 17 cases with ki-67 >15%, 11 (64.7%) of them highly express p27, this revealed a significant relationship (p value =0.043) between p27 expression and Ki-67 index. This is in agreement with those obtained by with Tsutsui *et al.* [24], they concluded that Bcl-2 protein expression closely correlated with p27, p53 protein expression and the proliferation activity determined by the MIB-1 counts in the breast.

CONCLUSION

There is a general agreement that high p27 levels are usually correlated with low tumour grade, positive oestrogen and progesterone receptor status and low Ki-67 index, however the prognostic role of p27 is not well established yet and requires further prospective studies with long term follow up and correlation with various patients, tumor parameters including genetic profiles.

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