# Immunohistochemical Expression of p21 in Ductal Carcinoma of the Breast and its Correlation with HER2/neu Expression and Hormonal Status

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**Abstract:** Breast cancer is the most common malignancy affecting women.It accounts for 23% of all cancers in women all over the world. In Egypt, breast cancer is the most common cancer among Egyptian women. Inspite of the improvements in early diagnostic methods of breast cancer and also the advances in treatment, mortality rate because of the disease remains high. The cyclin-dependant kinase inhibitor p21 (WAF1/CIP1) is a key regulator of progression from the G1 to the S-phase of cell cycle. This work aimed at studying the expression of p21 in invasive carcinoma of the breast in correlation with well-established prognostic factors in breast cancer including estrogen receptor, progesterone receptor status and HER2/neu expression. This study was carried out by retrieval of formalin-fixed, paraffin-embedded tissue sections from archival blocks prepared from 60 modified radical mastectomy specimens diagnosed as invasive duct carcinoma of the breast.The specimens were collected from the Department of Pathology, Kasr Al Aini Hospital School, Cairo University, Egypt from January 2011 till December 2012. Each case was tested for immunohistochemical expression of p21, estrogen receptors, progesterone receptors and HER2/neu. P21 expression was correlated significantly with the microscopic grade. Also, a highly significant relationship was found between p21 and expression of estrogen and progesterone receptors; moreover, loss of p21 was associated with positivity to HER2/neu. Our findings strongly suggest that p21 (WAF1/CIP1) expression may be used as a potential prognostic marker for human breast cancer, allowing therapy to be adjusted more appropriately for individual tumors.

**Key words:** Breast cancer %Estrogen receptors % HER2/neu% p21 % Progesterone receptors

## INTRODUCTION

Breast cancer is the most common malignancy that affects women, with a prevalence of 23% of all cancers in women worldwide. It is the second most common cause of death in women after lung cancer; almost one in every three affected women will die of the disease. The instance is high in more developed countries, whereas in less developed countries and in Japan, it is low but increasing. The median age is 50 years and the majority of cases (67%) in the west are postmenopausal [1]. In Egypt, carcinoma of the breast is the most prevalent cancer among Egyptian women and constitutes 17.5% of all malignant tumors presented to National Cancer Institute, Cairo University, in the years 2003-2004. The median age at diagnosis is one decade younger than that in countries of Europe and North America and most patients are premenopausal. Tumors are relatively advanced at presentation [2]. Despite improvements in early diagnostic methods and advanced in treatment, mortality because of breast cancer remains high and there are very little data on the factors that influence disease progression and mortality. Routine pathologic evaluation remains the most critical element in the determination of the prognosis of patients with breast cancer. Among the most important prognostic factors are tumor stage, histologic grade, histologic tumor type and lymphatic vascular invasion [3].

Invasive ductal carcinoma, the most common type of breast cancer, is a heterogeneous group of tumors that fail to show sufficient characteristics for classification as a specific histological type [4]. Biological immunoh istochemical markers are now being used widely as prognostic and predictive indicators in breast cancer including markers for estrogen receptors (ERs), progesterone receptors (PRs) and c-erb-B2 (HER-2/neu) [5]. Other new prognostic markers such as p21WAF1/CIP1 are becoming valuable tools for the prediction of prognosis and survival outcomes in women

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with breast cancer [6]. Cell cycle progression is regulated by cyclin-dependent kinases (CDKs) associated with cyclin proteins. P21WAF1/CIP1, a downstream target of p53, is a CDK inhibitor that activates p53-mediated G1 and G2 arrest following genotoxic insults, to facilitate DNA repair [7-10]. The integrity of G1 and G2 checkpoints requires the nuclear localization of p21WAF1/CIP1 [6, 10]. Recent evidence including subcellular fractionation suggests that p21WAF1/CIP1 can lodge in the cytoplasm in cancer tissues and cell lines, where it inhibits apoptosis by binding and inhibiting the apoptosis signal-regulating kinase 1 [11-12]. Such an anti-apoptotic function in breast cancers could underlie the association between cytoplasmic p21WAF1/CIP1 and poor prognosis [12]. Upregulation of p21WAF1/CIP1 occurs through PI-3K/Aktsignaling and may involve insulin-like growth factors, p53-dependent pathways of HER-2 expression [13]. An HER-2-overexpressing breast cancer cell line transcriptionally upregulates p21WAF1/CIP1, has been shown to produce its cytoplasmic localization through a mechanism whereby Akt binds and phosphorylated p21 WAF1/ CIP1in its nuclear localization signal [14]. *In vivo* HER-2 expression may involve changes in the subcellular localization of p21 (WAF1/CIP1) to affect the outcome in breast cancer.

Breast cancers with higher levels of cytoplasmic p21 (WAF1/CIP1) predicted reduced overall survival (OS) and relapse-free survival (RFS), with correlation between cytoplasmic p21WAF1/CIP1 and p53 expression. Recent in vitro findings demonstrated a direct influence of HER-2 on cytoplasmic p21 (WAF1/CIP1) [12]. HER-2 is one of the four Erb B family-type I receptor tyrosine kinases and is preferred dimerization partner for the epidermal growth factor receptor [15]. The Erb B receptors are important in the normal development and in human cancer. HER-2, independent of its own ligand, activates other Erb B receptors to increase their ligand affinity and to magnify biological responses. HER-2 have a key role in activating cytoplasmic signaling through the phosphatidylinositol-3 kinase (PI-3K)/protein kinase B (Akt) and mitogen activated protein kinase pathways to influence transcription of nuclear genes [16]. Activation of PI-3K/Akt is involved in cell proliferation and delivers resistance to apoptosis [17]. Breast cancer is associated with down-regulated expression of HER-2, detectable as HER-2 amplification or protein overexpression identified in 10-40% of tumors. HER-2 overexpression is an indicator of poor prognosis and may predict tumor responses to hormone therapy and chemotherapy [18].

## MATERIALS AND METHODS

**Patients Data and Tissue Samples:** This was a retrospective study involving the retrieval of formalin-fixed paraffin-embedded tissue sections from archival blocks of 60 cases of breast ductal carcinoma collected from the Department of Pathology, Faculty of Medicine, Cairo University, from January 2011 till December 2012. In this study, patients are eligible for inclusion if they were diagnosed with breast duct carcinoma (not otherwise specified) (NOS) according to the WHO classification [4] and had undergone modified radical mastectomy to provide adequate histologic sections for the proper application of all immunohistochemical markers included in the study. Tumor grading was evaluated according to the Nottingham combined histological grade (Elston-Ellis modification of the Scarff Bloom Richardson grading system) [19]. Patients who received preoperative therapy including radiotherapy, chemotherapy and hormonal therapy were excluded from the study, in order to avoid aberrant expression or false-positive results of the immunohistochemical workup, to obtain the most accurate prognostic findings possible.

**Histopathologic Examination:** All specimens were formalin fixed in neutral formalin 10% and embedded in paraffin. Hematoxylin and eosin slides of each case were prepared from each paraffin block for proper evaluation of tumor type and grade.

**Immunohistochemical Examination:** Four positively charged slides were prepared from representative tumor blocks of each specimen and stained with primary monoclonal antibodies against estrogen receptors (mouse monoclonal, clone 1D5, ready to use; Dako), progesterone receptors (mouse monoclonal, clone PgR636, ready to use; DakoGlostrup, Denmark), HER-2/neu (rabbit polyclonal, dilution 1:250; Dako) and p21 (mouse monoclonal Ab-1;DAKO,Denmark). In this study, the biotin-streptavidin amplified system was used. Paraffin sections were cut by the microtome at 4Fm thickness and mounted on positively charged glass slides. Sections were placed in xylene overnight and then heated in an oven at 52°C for 15 min for deparaffinization; then they were brought to distilled water through 100%, 95% ethanol, two changes, 10 min each and incubated in 3% hydrogen peroxidase activity. Then, the slides were placed in an unsealed plastic container filled with

sufficientantigenretrieval solution. Then, the slides were placed in a microwave for 5 min. Sections were incubated with blocking serum in a phosphate-buffered solution for 20 min to suppress nonspecific binding of immunoglobulins.One to two drops of the primary antibodies were placed on the sections. Slides were incubated overnight at 4°C; one to two drops of biotinylated secondary anti-immunoglobulins were applied for 30 min and incubated horizontally in humid chamber at room temperature. One or two drops of performed avidinbiotinylated horseradish peroxidase complex were applied for 30 min at room temperature. The antigen was finally localized with the addition of an appropriate precipitating chromogenic substrate; the chromogendiaminobenzidinetetrahydrochloride was used for 10-40 min until a brown color was obtained. Then the slides were washed in distilled water. Counterstaining was performed using Mayer's hematoxylinfor 3-5 min.

**Evaluation of Immunohistochemical Results:** Hormonal Status: ER and PR status were evaluated according to the Allred scoring system considering only nuclear staining [20] (Table 1).

**Evaluation of HER2/neu Status:** HER2/neuimmunostaining results were estimated according to the HER2/neu scoring system used to evaluate the HercepTest [21] (Table 2).

**Evaluation of p21:** P21 was considered positive only if nuclear staining was observed. Scoring of p21 immunostaining was performed using two main parameters including the percentage of p27 expression as well as the intensity of nuclear staining.The most cellular area of the tumor, with minimal necrosis or inflammatory cell infiltration and the highest nuclear labeling density, was selected and the number of positively stained nuclei was recorded in consecutive fields at x 400 magnification. The percentage of tumor nuclei expressing p27 was determined by counting 1000 cells/slide.The intensity of nuclear staining in the three-tier system was faint, moderated and strong. The area of the tumor showing the highest burden of nuclear expression was used for evaluation. P21 nuclear localization in association withlymphocytes as well as normal breast lobules was taken as a reference for strong nuclear staining intensity. Cytoplasmic localization was also reported if present. On examination of cases with an in situ component, immunohistochemical results of only invasive component were included in the statistical analysis.

Table 1: Allred score for the evaluation of estrogen receptor and progesterone receptor staining.

| Proportion score | Positive cells (%) |
|---|---|
| 0 | 0 |
| 1 | <1 |
| 2 | 1-10 |
| 3 | >10-33 |
| 4 | >33-66 |
| 5 | >66 |
| Intensity score | Intensity of positivity |
| 0 | None |
| 1 | Weak |
| 2 | Intermediate |
| 3 | Strong |
| Total score | Interpretation |
| 0,2 | Negative |
| 3,4,5,6,7,8 | Positive |

Allred *et al*. [20]

Table 2: HER2/neu score used to evaluate Hercep test.

| Score | Criteria |
|---|---|
| 0 (negative) | No immunoreactivity in <10% of tumor cells |
| 1+ (negative) | Faint weak immunoreactivity in >10% of tumor cells but only a portion of the membrane is positive (incomplete) |
| 2+ (weak positive) | Weak to moderated complete membrane immunoreactivity in >10% of tumor cells |
| 3+ (positive) | Moderate to strong complete immunoreactivity in >10% of tumor cells |

Jacobs *et al*. (21)

**Statistical Analysis:** Data were statistically described in terms of range, mean ±SD, median, frequencies (numbers of cases) and percentages when appropriate. Comparison of quantitative variables between the study groups was carried out using the Mann-Whitney U-test for independent samples for the comparison of two groups and the Kruskal-Wallis analysis of variance test for the comparison of more than two groups. Within-group, comparison of quantitative variables was carried out using the Wilcoxon signed rank test for paired (matched) samples. For comparison of categorical data, the $x_2$ test was performed. Exact Fisher's test was used when the expected frequency was less than 5. Correlation between the different study variables was determined using the Spearman correlation equation for non-normal variables. *P* value less that 0.05 was considered statistically significant. All statistical calculations were carried out using the computer programs Microsoft Excel 2003 (Microsoft Corporation, New York, New York, USA) and statistical package for the social science (SPSS; SPSS Inc., Chicago, Illinois, USA), version 15 for Microsoft Windows.

## RESULTS

This study included 60 cases of ductal carcinoma collected from archival blocks present in the Pathology Department, Cairo University, Kasr Al Aini Hospital (Figs 1 and 2). The histopathologic characteristics and immunohistochemical results of all the groups studied are presented in Table 3. The mean age of patients was 56.2 years, with a median of 56 years, ranging from 27 to 86 years. The highest incidence occurred in patients equal to or older than 50 years of age, comprising 56.7%, whereas 43.3% of the patients were <50 years of age. All of the 60 cases studied were diagnosed with invasive ductal carcinomas. Extensive DCIS has been proposed for tumors in which the intraductal component comprises 25% or more of the area encompassed by the infiltrating tumor and is also present in the surrounding breast tissue [22]. A total of 50% were associated with a DCIS component <25% of the tumor area (minor DCIS component) and 15% were associated with major DCIS. The remaining cases of invasive duct carcinomas were not associated with the DCIS component, comprising 35% of all the cases studied. In terms of tumor grade, 29 cases (48.3%) were grade II and 31 cases (51.7%) were grade III. None of the cases studied were grade I.

**Immunohistochemical Results:** Estrogen and Progesterone Receptor Staining: Among the 60 cases studied 80% showed positive staining for ER, whereas only 20% were negative (Fig. 3). Immunohistochemical staining for PR indicated 73.3% positive cases and 26.7% negative cases. One case only showed ER negative tumor receptorsand positive PR receptors in the same time. On the other hand, 11 cases (18.3%) were negative for both ER and PR tumor receptors.

**HER2/neuImmunostaining:** Forty-one out of 60 cases (68.3%) showed negative staining to Her2/neu (score 0 and 1+), whereas positive (score 3+) and equivocal (score 2+) results were obtained in 15 (25%) and in 4 (6.7%) cases, respectively (Figs 4 and 5).

**P21WAF1/CIP1 Immunostaining Results:** In this study, we have estimated p21 expression in terms of three main parameters: percentage of positive nuclear-stained cells, intensity of nuclear staining and the presence or absence of cytoplasmicmislocalization among tumor cells (Fig. 6 and 7 and 8).

Table 3: Histopathologic and immunohistochemical characteristics of the studied cases.

| Item | N% |
|---|---|
| Age | |
| < 50 | 26 (4303) |
| $ 50 | 34 (56.7) |
| Total | 60 |
| Histologic type | |
| IDC | 21 (35) |
| Invasive duct carcinoma with minor DICS | 30 (50) |
| Invasive duct carcinoma with major DICS | 9 (15) |
| Total | 60 |
| Grade of IDC | |
| 2 | 29(48.3) |
| 3 | 31(51.7) |
| Total | 60 |
| Hormonal status | |
| Positive hormone receptor(s) | 49 (81.7) |
| ER+ and PR+ | 43 (71.7) |
| ER+ and PR- | 5 (8.3) |
| ER- and PR+ | 1 (1.7) |
| Negative hormone receptor(s) | |
| ER- and PR- | 11(18.3) |
| Total | 60 |
| HER2 expression | |
| Strong positive (score 3+) | 15 (25) |
| Negative (score 0 and 1+) | 41 (68.3) |
| Weak positive (score 2) | 4 (6.7) |
| Total | 60 |
| P27 percentage at 25% cut off point | |
| High expressors (> 25%) | 34 (56.7) |
| Low expressors (# 25%) | 26 (43.3) |
| Total | 60 |
| P27 nuclear intensity | |
| Faint | 33 (55) |
| Moderate | 21 (35) |
| Strong | 6 (10) |
| Total | 60 |
| Cytoplasmic localization of p27 | |
| Positive | 41 (68.3)) |
| Negative | 19 (31.7) |
| Total | 60 IDC, |

invasive duct carcinoma; DICS, ductal carcinoma in situ; ER, estrogenreceptors; PR, progesterone receptors.

**Percentage of p21:** All cases expressed positive nuclear staining for p21, with variable percentages ranging from 11 to 97.3%. The median percentage among all the cases examined was 66%. It was observed that the in-situ component showed higher p21 expression than the invasive component within the same case.

In this study, the 25% level was considered as a convenient cut-off point, so that those tumors recording less than or equal to 25% positive cells for p21 were

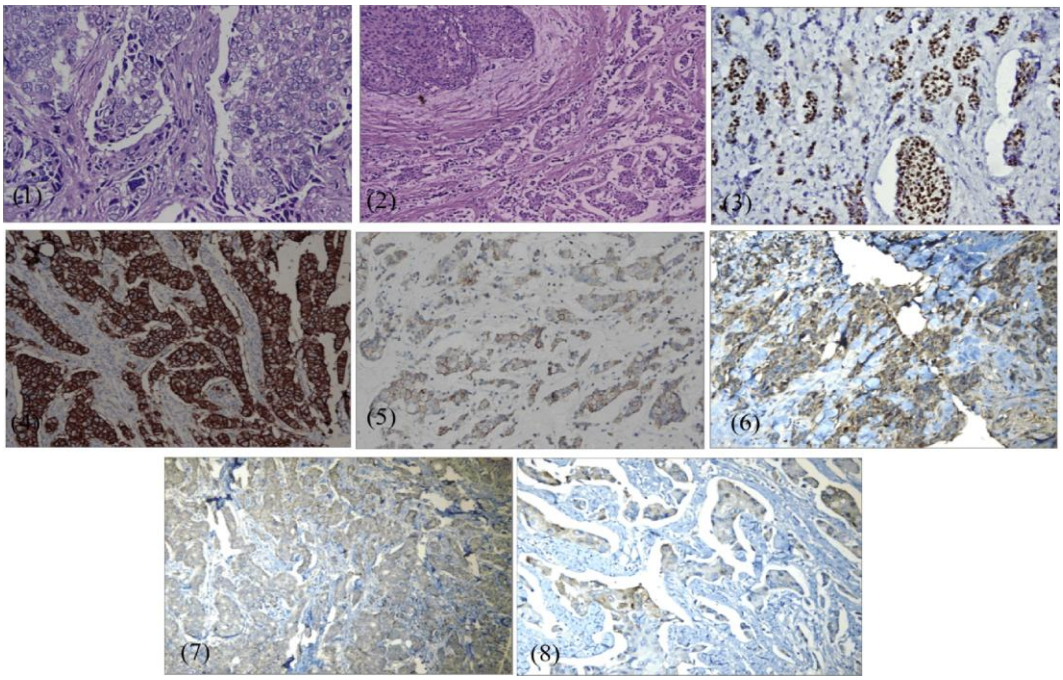


Fig. 1: Invasive duct carcinoma grade III (H&E x400)
Fig. 2: Invasive duct carcinoma grade II with in situ component (H&E x200)
Fig. 3: Invasive duct carcinoma grade III showing positive nuclear staining for estrogen receptor (ER) with a strong intensity and high proportion (x400)
Fig. 4: Invasive duct carcinoma grade III with strong complete membranous immunoreactivity to HER2/neu (x400)
Fig. 5: Invasive duct carcinoma grade II with incomplete membranous immunoreactivity to HER2/neu (x400)
Fig. 6: Invasive duct carcinoma grade III with a moderately positive nuclear intensity for p21 and high expression (92%) (x200)
Fig. 7: Invasive duct carcinoma grade III with cytoplasmic localization of p21 (x200)
Fig. 8: Invasive duct carcinoma grade III with cytoplasmic localization of p21 and low-expression (11%) of faint, rare p21 nuclear staining (x400)

considered low expressors (n=26), whereas those expressing more than 25% positive cells for p21 were considered high expressors (n=34). This cut-off point was chosen as most of the significant correlations were obtained on comparing the p21 score at 25% level [23, 24].

**Intensity of Nuclear Staining:** Thirty-three out of the 60 cases (55%) examined showed faint nuclear staining for p21, whereas only 10% showed a strong intensity. A moderated intensity of p21 staining was found in 35%. Also, the intensity of nuclear staining was higher in the in-situ component that in the invasive one within the same case.

**Cytoplasmic Mislocalization:** In terms of the presence of p21 cytolplasmicmislocalization, 41 out of 60 cases (68.3%) showed cytoplasmic mislocalization of p21.

**Correlation between p21 Expression and Tumor Grade and Extent of the *in situ* Component:** There was a highly statistically significant relation between p21 expression and grade of tumorin most of the parameters (Table 4). In terms of the percentage of p21 the majority (90.3%) of grade III tumors showed high p21 nuclear expression, whereas the majority (79.3%) of grade II tumors were in the low-expression category ($p<0.001$). According to p21 intensity, a borderline significance was found in relation to tumor grade. More than half (62.1%) of grade II tumors showed a faint nuclear staining intensity, whereas only 12.9% of grade III tumors showed a strong intensity ($p=0.052$). A highly statistically significant correlation was found between cytoplasmic mislocalization of p21 and tumor grade. The majority (87.1%) of grade III tumors showed positive cytoplasmic mislocalization whereas 46.3% of grade II tumors showed positive cytoplasmic

Table 4: Correlation between p21 expression and tumor grade.

| | N (%)1 | | | | | | |
|---|---|---|---|---|---|---|---|
| | Percentageof p21 | | Intensity of nuclearstaining | | | Cytoplasmiclocalization | |
| Tumor grade | Highexpressors | Lowexpressors | Faint | Moderate | Strong | Negative | Positive |
| Grade II | 6 (20.7) | 23 (79.3) | 18 (62.1) | 9 (31) | 2 (6.9) | 15 (51.7) | 14 (46.3) |
| Grade III | 28 (90.3) | 3 (9.7) | 15 (48.4) | 12 (38.7) | 4 (12.9) | 4 (12.9) | 27 (87.1) |
| Total | 34 (56.7) | 26 (43.3) | 33 (55) | 21 (35) | 6 (10) | 19 (31.7) | 41 (68.3) |
| Significant | P < 0.001 | | P = 0.052 | | | P = 0.001 | |

Table 5: Correlation between p21 expression and hormone status.

| | N (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Percentageof p21 | | Intensity of nuclear staining | | | Cytoplasmic localization | |
| Hormone status | Highexpressors | Lowexpressors | Faint | Moderate | Strong | Negative | Positive |
| Estrogen receptors | | | | | | | |
| Negative | 6 (50) | 6 (50) | 8 (66.7) | 4 (33.3) | 0 (0) | 4 (33.3) | 8 (66.7) |
| Positive | 28 (58.3) | 20 (41.7) | 25 (52.1) | 17 (35.4) | 6 (12.5) | 15 (31.3) | 33 (68.8) |
| Total | 34 (56.7) | 26 (43.3) | 33 (55) | 21 (35) | 6 (10) | 19 (31.7) | 41 (68.3) |
| Significant | P = 0.002 | | P <0.001 | | | P = 0.021 | |
| Progesterone receptors | | | | | | | |
| Negative | 8 (50) | 8 (50) | 9 (56.3) | 7 (43.8) | 0 (0) | 6 (62.5) | 10 (62.5) |
| Positive | 26 (59.1) | 18 (40.9) | 24 (54.5) | 14 (31.8) | 6 (13.6) | 13 (29.5) | 31 (70.5) |
| Total | 34 (56.7) | 26 (43.3) | 33 (55) | 21 (35) | 6 (10) | 19 (31.7) | 41 (68.3) |
| Significant | P = 0.004 | | P <0.001 | | | P = 0.053 | |

Table 6: Correlation between p21 expression and HER2/neu.

| | N (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Percentageof p21 | | Intensity of nuclearstaining | | | Cytoplasmiclocalization | |
| HER2/neu | Highexpressors | Lowexpressors | Faint | Moderate | Strong | Negative | Positive |
| Positive | 7 (46.7) | 8 (53.3) | 7 (46.7) | 8 (53.3) | 0 (0) | 3 (33.3) | 10 (66.7) |
| Negative | 24 (58.5) | 17 (41.5) | 23 (56.1) | 12 (29.3) | 6 (14.6) | 14 (34.1) | 27 (65.9) |
| Equivocal | 3 (75) | 1 (25) | 3 (75) | 1 (25) | 0 (0) | 0 (0) | 4 (100) |
| Total | 34 (56.7) | 26 (43.3) | 33 (55) | 21 (35) | 6 (10) | 19 (31.7) | 41 (68.3) |
| Significant | P = 0.001 | | P <0.001 | | | P = 0.037 | |

localization (p=0.001). No statistically significant correlation was found between the extent of DCIS and p21 expression including p21 percentage, intensity of nuclear staining and cytoplasmic mislocalization, yielding p-values of 0.321, 0.215 and 0.894, respectively.

**Correlation between p21 Expression and Hormonal Status:** Data presented in Table 5 shows a statistically significant correlation was found between estrogen and progesterone status and p21 expression at the level of all the parameters included for the evaluation of p21.In terms of correlation with p21 percentage, the majority of high expressor tumors (82.4%) were ER-positive (p=0.002).simultaneously, 76.5% of high expressortumors were PR-positive (p=0.004). In terms of the nuclear intensity of p21, all tumors with a strong intensity (100%) were ER-positive (p<0.001) and PR-positive (p<0.001).Considering the cytoplasmic localization of p21, the majority of tumors with negative cytoplasmic staining (78.94%) were positive for ER (p=0.021) and also 68.42% of cases negative to cytoplasmic staining for p21 were positive for PR (p=0.053).

**Correlation between p21 Expression and HER2/neu Expression:**Data in Table 6 shows a statistically significant correlation was found between HER2/neu expression and all the parameters used to evaluate p21 expression.In terms of the percentage of p21 positive cells 70.58% of tumors with a high expression of p21 were negative to HER2/neu (p=0.001). In terms of nuclear expression, 100% oftumors showing strong nuclear expression for p21 were negative to HER2/neu (p<0.001).The majority of HER2/neu positive tumors (77%) showed the presence of p21 cytoplasmic mislocalization (p=0.037).

## DISCUSSION

Breast cancer is the most common cancer affecting women in the world today with a prevalence of 23% of all cancers in women. It is the leading cause of cancer related death of women aged between 35-65 years worldwide. More than a million women are diagnosed with breast cancer every year, accounting for a tenth of all new cancers and ranks second overall (10.9% of all cancers) [1]. Despite improvement in early diagnostic methods and advances in treatment, mortality because of breast cancer remains high; there are small data on the factors that influence disease progression and mortality. New prognostic factors such as P21 are becoming valuable tools for the prediction of prognosis and survival outcomes in women with breast cancer [24]. P21WAF1/CIP1, a downstream target of p53, is a CDK inhibitor that re-enforces p53-mediated G1 and G2 arrest the following genotoxic insults, to facilitate DNA repair [7-10]. Recent evidence including subcellular fractionation suggests that p21WAF1/CIP1 can localize in the cytoplasm in cancer tissues and cell lines, where it inhibits apoptosis. Such an anti-apoptotic function in breast cancers could underlie the association between cytoplasmic p21WAF1/CIP1 and poor prognosis [12]. HER-2-overexpressing breast cancer cell line transcriptionally upregulates p21WAF1/CIP1 and has been shown to produce its cytoplasmic localization [14]. A better understanding of the mechanism regulating p21 expression and its interaction with other oncogenes provides the possibility of selective control of its degradation, which may facilitate the generation of new and more effective drugs for breast cancer [12]. In this work, we aimed to study the expression of p21 intraductal carcinoma, which is the most common type of breast carcinoma and to evaluate the relation between p21 and other important well established prognostic parameters of breast carcinoma including hormonal status as well as HER2/neu expression.

We included 60 cases of ductal carcinoma collected from archival blocks from the Pathology Department, Faculty of Medicine, Cairo University. The mean age of the patients with breast carcinoma studied was 56.2 years and the median was 56 years, ranging from 27 to 87 years; the majority of the cases (51.7%) were of grade III. None of our cases had received any kind of neoadjuvant therapy to ensure accurate estimation of immunohistochemical results. In this study, we estimated p21WAF1/CIP1 among the cases studied in terms of three main parameters: percentage of positive cells, intensity of nuclear staining and the detection of cytoplasmic mislocalization. All our cases showed expressed positive nuclear staining for p21 with variable percentages ranging from 11% to 97.3 %, with a median percentage 66 %. The majority of cases (56.7%) were in the high expression category, with more than 50% showing positive nuclear staining. In terms of the intensity of nuclear staining, most of the cases (55%) showed a faint staining intensity. In our study, 41 of 60 (68.3%) cases showed cytoplasmic mislocalization.

It is a worth noted that we observed higher p21 expression in the in situ component than the invasive component in the same case. This strongly suggests that the loss of p21 plays a role in the progression of breast cancer. Jiang *et al.* [23] observed a frequent loss of p21 in noninvasive ductal breast carcinoma insituand, in both the insitu and the invasive components; lower p21 staining was observed in high-grade tumors.

We found a highly statistically significant difference between p21 expression and grade of tumor.90.3% of grade III tumors showed high p21 nuclear expression, whereas 79.3% of grade II tumors were in the low expression category (p<0.001). Moreover, in terms of p21 nuclear staining intensity, more than half (62.1%) of grade II tumors showed a faint nuclear intensity, whereas only 12.9% of grade III tumors showed a strong intensity (p=0.052).Also, in terms of cytoplasmic mislocalization, 87.1% of grade III tumors show positive cytoplasmic staining for p21 while more than half (51.7%) of grade II tumors show negative cytoplasmic staining (p=0.001). Our results were in agreement with those obtained by Caffo *et al.* [25] and Pellikainen*et al.*[26], who found an increasing incidence for p21 expression from grade I tumors till grade III tumors with a weak and faint staining intensity (p=0.001 and 0.017, respectively). This could be

attributed to the fact that as the grade of tumor becomes higher; p21 staining shows transition from the nucleus to mislocalize in the cytoplasm. In terms of hormonal status, we obtained astatistically significant relation between p21 expression and positivity to both estrogen and progesterone receptors. In terms of correlation with p21 percentage, the majority of high expressortumors (82.4%), were ER-positive (p=0.002). Simultaneously, 76.5% of high expressortumors were PR-positive (p=0.004).

In terms of nuclear intensity of p21,all the tumors with a strong intensity (100%) were ER-positive (p<0.001) and PR-positive (p<0.001).These results were in agreement with those obtained by Pellikainen*et al.* [26] and Winters *et al.* [27] that indicated a statistically significant relationship between p21 expression and hormone receptor status in breast cancer (p=0.077 & 0.05 respectively).In addition, Chen *et al.* [25] found a strong association between low P21 and loss of hormone receptors (p<0.001) and finally concluded that loss of p21 may lead to worse prognosis. In a cell with functional ERs, estrogen mediates the transition of cells from the G1 to S phase of the cell cycle; antiestrogens inhibit ER signaling and arrest cells in the G1 phase [28, 29]. Several studies [30-33] have shown that cyclin D1, a G1 cyclin, can interact directly with the ligand-binding domain of ER and stimulate transactivation of ER in a ligand-independent and cyclin- dependent kinase (CDK)-independent fashion. P21WAF1/CIP1is a major negative regulator of the G1 checkpoint by binding to and inhibiting the activities of most cyclin/CDK complexes. In addition, p21 can function as adaptor molecules that promote the association of CDK4 with D-type cyclins and that increase CDK4 kinase activity [34-35]. P21 may also be involved in G1-phase arrest mediated by antiestrogenic inhibition of ER. In fact, transfection of ER or p21 complementary DNAs (cDNAs) into breast cancer cells results in growth inhibition or apoptosis of the transfected cells. Because ER and p21 have growth-regulatory effects in normal and tumor cells, we discovered that p21 can act as a mediator of estrogenic actions, sensitizing these cells to the growth inhibitory effects ofantiestrogens[36-37]. The p21-mediated activation of the estrogen-signaling pathway has important clinical implications. A number of agents currently used for the treatment of breast cancer result in the induction of p21. For example, paclitaxel (Taxol), a potent and highly effective antineoplastic agent for the treatment of advanced, drug-refractory, metastatic breast cancers, results in the induction of p21 in the ER-negative MDA-MB-435 cell line [38]. Similarly, treatment of MCF-7 cells with paclitaxel [39],with doxorubicin in the presence or absence of neu differentiation factor [40],or with vinorelbine (a vinca alkaloid analogue) in combination with a progesterone analogue results in the induction of p21 [41]. Last, treatment of a number of breast cancer cell lines and primary cultures from 20 different invasive ductal carcinoma of the breast with doxorubicin resulted in a substantial increase in the level of p21 [42]. Similar to chemotherapeutic agents, several differentiationinducing agents also result in the induction of p21. For example, p21 is induced in HL60 leukemia cells by agents, such as vitamin D3 [43],12-*O*-tetradecanoylphorbol 13-acetate, retinoic acid, dimethyl sulfoxide [44] and butyrate. P21 is induced in melanoma cells when cells are terminally differentiated with recombinant human fibroblast interferon and mezerein [45]. P21 is induced in leukemia cells by okadaic acid [46]. Consequently, it follows that chemotherapy of ER-negative breast cancer may activate the estrogen-signaling pathway. The clinical translation of this hypothesis lies in the potential of p21-positive, ER-negative breast cancers to respond to anti-estrogens. These results suggest a treatment strategy that combines chemotherapy and antihormonal therapy for ER-negative cancers that express p21. Because chemotherapeutic agents can induce p21 and p21 can activate the estrogen pathway, such a combination therapy could benefit patients with ER-negative breast cancer that do not respond to anti-estrogens. Induction of the estrogen-signaling pathway by increased levels of p21 could ultimately promote tumor cell differentiation and result in tumor regression and improved survival rates. This treatment strategy could also benefit patients with ER-negative, progesterone receptor-negative breast cancers that have high basal levels of p21.Consequently, ER-positive, PR positive breast cancers that high basal levels of p21 can have more benefit and extended survival rates with such drugs in combination with Tamoxifen (anti-estrogen) as an adjuvant treatment [47].

In our study, we found a significant correlation between p21 and HER2 immunostaining. Loss of p21 was found to be associated with positivity to HER2/neu. In terms of the percentage of p21-positive cells, 70.6% of tumors with a high expression of p21 were negative to HER2 (P=0.001). In terms of nuclear expression, 100% of tumors showing a strong nuclear intensity for p21 were negative to HER2 (P<0.001). Our significant results were in complete agreement with those of Xia *et al.* [24], who found that all 42 (100%) of the tumors that overexpressed HER2/neu had low levels of p21 protein product; this

correlation was significantly significant (p<0.001).They also reported that decreasing p21 expression is correlated with increasing HER2/neu activity and finally concluded that one function of the HER2/neu product is to downregulate p21 expression in breast cancer and may be significant in selecting patients for HER2/neu antibody therapy in the future. Cytoplasmic localization of p21 was observed in 68.3% of our studied cases. Interestingly, we found a significant correlation between cytoplasmic localization of p21 and hormonal status. The majority of tumors with no evidence of cytoplasmic staining (79%) were positive for ER (P=0.021) and also (68.4%) of cases negative to cytoplasmic staining for p21 were positive for PR (P=0.053).Moreover, the presence of cytoplasmic localization was associated with HER2/neu positivity. The majority of HER2/neu-positive tumors (77%) showed the presence of p21 cytoplasmic mislocalization (p=0.037). The biological implications of cytoplasmic p21WAF1/CIP1 with respect to the ability to assemble and inhibit cyclin/CDK complexes and to bind apoptosis signal-regulating kinase 1 require further investigation. Studies in cell culture suggest a loss of p53 and growth suppressor function, with inhibition of apoptosis. Subcellular localization of p21WAF1/CIP1may have relevance underlying mechanisms of HER-2 drug resistance, with the potential for cytoplasmic p21WAF1/CIP1-expressing breast tumors to have increased chemo-resistance or hormone resistance. Small patient numbers precluded a separate analysis of treatment relapses in cytoplasmic p21WAF1/CIP1-expressing tumors. The ability of HER-2 to dysregulatep21WAF1/CIP1, a key target of p53, is highly relevant in the context of p53 wild-type cancers in which growth suppression and apoptosis may be inhibited through mislocalizationpfp21WAF1/CIP1[27].

## CONCLUSION

These presented findings indicate that HER-2 expression may influence tumor outcome through a mechanism regulating the subcellular localization of p21WAF1/CIP1 to produce a cytoplasmic distribution resulting in a loss of its tumor suppressor functions. Cytoplasmic p21WAF1/CIP1 predicts poor prognostic tumors and may have a role in HER-2-mediated drug resistance.In conclusion, our results strongly point to the role of p21WAF1/CIP1 in carcinogenesis of breast carcinoma related to established prognostic factors, including tumor grade, hormonal status and HER2/neu expression. We suggest that p21WAF1/CIP1 plays an important role as a prognostic factor and can identify a subset of breast cancer patients with a poor prognosis, who require different therapeutic interventions.We strongly recommend and encourage thorough and large scale studies to establish a reliable consensus for scoring methods for the evaluation of p21 to formulate a standardized technique for immunostaining and scoring that can be used widely similar to that of HER2/neu. Further studies should be carried out that correlate p21 with the prognosis of breast cancer and prediction of response to therapy through integration into many clinical trials in order to identify the necessity to involve p21 as a routinely tested marker in newly diagnosed breast cancer patients and become as evidence based as hormonal status and HER2/neu.

**Conflicts of Interest:** There are no conflicts of interest.

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