Role of Ki67 in predicting resistance to adjuvant tamoxifen in postmenopausal breast cancer patients

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Breast cancer; Adjuvant hormonal therapy; Tamoxifen; Postmenopausal; Ki67; National Cancer Institute; Egypt

Abstract
Introduction: Breast cancer (BC) is a major health problem in Egypt and worldwide. Its prognosis depends not only on tumor stage but also on tumor biology.
Aim: To correlate the expression of Ki67 with the clinical outcomes of early hormone-receptor positive postmenopausal BC patients who are receiving tamoxifen.
Methods: This cohort study included 70 patients. They were followed up for a minimum of 2 years. Ki67 was assessed on paraffin-embedded blocks using immunohistochemistry methods.
Results: The median Ki67 value was 22.5% (IQR, 10%–50%). Ki67 was significantly higher in patients with HER2 positive tumors compared to HER2 negative tumors. After a median follow up period of 53 months, 22 patients (31%) developed disease recurrence either loco-regional or distant in 5.7% and 30%, respectively. Recurrent patients had significantly higher tumor stage, nodal stage and Ki67 values compared to non-recurrent cases. The 2-, 3- and 5-year overall survival (OS) and disease-free survival (DFS) rates were 100% & 91%, 98% & 84% and 77% & 59%, respectively. DFS was significantly worse with higher TNM stage, lower ER expression and higher Ki67 values. OS was significantly worse in patients with Ki67 values ≥ 30%. Ki67 ≥ 30% was an independent predictor of recurrence, poor DFS and OS.
Conclusion: High Ki67 expression is predictive of poor prognosis and of resistance to adjuvant tamoxifen therapy in postmenopausal BC. We recommend considering Ki67 as one of the risk factors that guide adjuvant treatment decisions.

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Introduction
Breast cancer (BC) is the most common female malignancy accounting for 22.9% and 37.7% of all female cancers worldwide and in Egypt, respectively [1]. These estimates are confirmed in many regional Egyptian cancer registries [2,3] as well as in hospital-based frequencies [4]. BC is also the leading cause of cancer death in females accounting for 13.7% of
female cancer-related mortality globally compared to 29.1% in Egypt. Incidence to mortality ratio of BC in Egypt is poor (1.9:1) compared to a more favorable global ratio (3.7:1) [1].

In developed countries, around 65% of all BC occurs in postmenopausal women, of which about 80% are hormone receptor-positive [5,6]. In Egypt, 51% of breast cancers occur in postmenopausal women of whom 60% are hormone receptor-positive [7]. The anti-estrogen tamoxifen is the drug most often used for the long-term treatment of early breast cancer [8]. Tumor recurrence and mortality are significantly decreased by the use of 5 years of adjuvant tamoxifen both in the presence and absence of adjuvant chemotherapy [9]. However, a serious limitation of tamoxifen is the inevitable appearance of resistance; either de novo or acquired [8]. Approximately 40% of the patients receiving adjuvant tamoxifen therapy experience tumor relapse and die from their disease [10]. The yearly recurrence rates remain above 2% for a long period and more than 30% of women develop recurrent disease within 15 years [9].

Tamoxifen competes with estrogen for estrogen receptors (ERs) leading to conformational changes that prevent binding of co-activators and blocks activating function-1 (AF-1) domain [10]. The proposed mechanisms of tamoxifen resistance are diverse and include: Loss of ERα expression/function [11], altered expression of ERβ [12], lack of PR expression [13], alterations in co-regulatory proteins [14], decreased cellular influx or increased efflux [15], pharmacogenetic effects particularly those involving CYP2D6 or cross talk between ER and other signal transduction pathways [16].

Ki67 is a nuclear protein expressed during cellular proliferation particularly during the mitosis phase [17]. In early BC, high Ki67 is an independent factor for worse prognosis as shown by significantly shorter overall and disease-free survival [18,19]. High Ki67 was predictive of more benefit from adjuvant letrozole over tamoxifen [20] and adjuvant taxane over non-taxane regimens [21,22]. On the other hand, Ki67 was not predictive of more benefit of adjuvant chemoendocrine therapy over endocrine therapy alone [23] or adjuvant epirubicin-CMF over CMF [24].

The aim of the current study was to correlate the expression of Ki67 with the clinical outcomes of early hormone-receptor positive postmenopausal breast cancer patients who are receiving adjuvant tamoxifen.

Methods

Design and setting

This was a prospective cohort study conducted in the Medical Oncology Department, National Cancer Institute, Cairo University (NCI-Egypt) between July 2007 and March 2012. The study was conducted in compliance with Good Clinical Practice guidelines and the ethical principles routed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of NCI-Egypt.

Eligibility

Patients were included in the study if they were postmenopausal females, had histologically confirmed BC, had hormone receptors’ positive (HR) tumors (i.e. positive for estrogen receptors [ER] and/or progesterone receptors [PR]), undergone curative surgery, and were receiving adjuvant tamoxifen. Patients were excluded if they were males or premenopausal females, negative for ER and PR, or receiving aromatase inhibitors. Sampling was consecutive.

Immunohistochemistry (IHC) studies and their interpretation

Formalin-fixed paraffin-embedded blocks of all patients were obtained from the pathology department of NCI-Egypt for IHC study. One 4-µm section of each submitted paraffin block was first stained with Hematoxylin and Eosin to verify that an adequate number of invasive carcinoma cells were present, and the fixation quality was sufficient for IHC analysis. ER & PR were evaluated using Monoclonal Antibody, Ready-to-Use (Cell Marque, USA). HER2 was evaluated by using Mouse Monoclonal Antibody, Ready-to-Use (Clone e2-4001+3B5, Cat.#MS-730-R7, Thermo Scientific, USA). Ki67 was evaluated using Rabbit Polyclonal Antibody, Ready-to-Use (Cat.#RR-9043-R7, Thermo Scientific, USA). p53 was evaluated by using a Mouse Monoclonal Antibody, Ready-to-Use (DO7, Cat.#453M-98, Cell Marque, USA). The universal kit used was Anti-Polyvalent, HRP/DAB, Ready-to-Use (Cat.#TP-015-HD, Thermo Scientific, USA).

According to recommendations of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP), ER and PR were considered positive for expression when ≥1% of the cell nuclei stain with the antibody [25]. Interpretation of HER2 was also according to the recommendations of ASCO/CAP with a positive HER2 result being IHC staining of 3+ (uniform, intense membrane staining of >30% of invasive tumor cells) [26]. According to the recommendations of the International ki67 in Breast Cancer Work group, the Ki67 score was defined as the percentage of total number of tumor cells with nuclear staining by the antibody [27]. Ki67 was regarded as overexpressed at score ≥14% [28]. p53 expression was determined by the percentage of total number of tumor cells with nuclear staining by the antibody & was regarded as accumulated (positive for overexpression) when ≥5% of nuclei were stained, whereas values <5% were regarded as negative for mutation [29].

Statistical issues

All analyses were done using SPSS® (Statistical Package for Social Sciences) software, version 17, Chicago, USA. Numerical values were expressed as means or medians and standard deviation (SD) or range. Survival was estimated using product-limit methods. Curves were compared using log rank test. Correlations between numerical variables were assessed using the Pearson’s correlation method. Cox regression analysis depicted independent covariates on different disease outcomes. To be included in the regression analysis, a variable should have a p value ≤0.1. Proportions were tested for independence using the Chi-square and Fisher exact tests. A receiver operator characteristic (ROC) curve was the tool used to select a cutoff point for Ki67 as a continuous variable. P value was set significant at ≤0.05 level. The primary endpoint was disease-free survival (DFS). The secondary endpoint was overall survival (OS).
Definitions

DFS was defined as the time between the date of curative surgery and date of first relapse (local or distant) or death. OS was defined as the time between the date of diagnosis and date of death or lost to follow up. Time to recurrence (TTR) was defined as the time between the date of curative surgery and date of relapse. Early and late recurrences were defined as relapse within or more than 24 months from the start of adjuvant tamoxifen, respectively.

Results

Patients' characteristics and the corresponding Ki67 expression

The study included 70 patients with their characteristics shown in Table 1. The mean age ± SD was 60 ± 5 years. Most patients had T2 tumors of IDC histology and grade II. The median tumor size was 30 mm (range, 15–105). The majority of patients had node-positive and TNM stage III disease. Only 13 patients (18.6%) had HER2 positive disease and hence luminal B subtype. According to the study eligibility, all patients were hormone receptor (HR) positives (i.e. ER and/or PR+) and had curative surgery being modified radical mastectomy (MRM) in 89% of patients or breast conserving surgery (CBS) in 11% of them. While none received neoadjuvant hormonal therapy, 12 patients (17%) received neoadjuvant anthracycline-based chemotherapy; FAC in 7 patients and FEC in 5 patients. While adjuvant radiotherapy was used in 76% of patients, adjuvant chemotherapy was used in 86% of them; FAC in 39%, FEC in 36%, CMF in 10% and FEC-doxetaxel in 1%. None of the HER2 positive patients received trastuzumab. All patients received adjuvant tamoxifen for a median duration of 44 months (range, 9–60). The drug was generally well tolerated with no serious adverse events reported.

The median Ki67 value in the whole population was 22.5% (range, 1%–90%). Apart from a significantly higher Ki67 in HER2 positive than HER2 negative tumors (median, 35% vs. 22.5%, p < 0.01), no significant correlation was found with other clinicopathologic characteristics.

Table 1

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<th>Ki67 ≥ 14% no. (%)</th>
<th>p</th>
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null
be younger \((p = 0.07)\) with higher T stages \((p = 0.46)\) and to have more HER2 positive tumors and subsequently luminal B subtype \((p = 0.21)\). Relapse was significantly associated with Ki67 with both of the used cut-offs (Table 2). Therapeutic interventions (surgery, radiotherapy and chemotherapy) did not differ significantly between the two groups.

The median time to recurrence was 35 months (range, 17–57). The median time from tamoxifen start to recurrence was 27 months (range, 9–49). Of the 22 relapses, 10 relapses were encountered within \((\leq) 24\) months from tamoxifen start (early relapses) and 12 relapses started \(>24\) months from tamoxifen start (late relapses). Comparison between patients with early relapse and those with late relapse showed no significant differences in patients’ clinical or pathological characteristics or treatment patterns. Ki67 was significantly higher in patients with early relapse compared to the non-relapsed counterparts (median, 53% vs. 20%, range, 5%–90% vs. 1%–90%, \(p = 0.01\)). Patients with late relapse had a marginally higher median Ki67 than the non-relapsed counterparts (median, 35% vs. 15%, range, 5%–85% vs. 1%–90%, \(p = 0.08\)). The median Ki67 was not significantly different according to the time of relapse.

**Breast cancer subtypes**

Considering ER, PR and HER2, 57 tumors (81.4%) were classified as luminal A (LA) and 13 tumors (18.6%) as LB (Table 3). When Ki67 was additionally considered, 31 tumors (44.3%) that were originally considered as LA had a Ki67 value \(\geq 14\%\) and were subtracted from LA (now became 26 tumors [37.1%]) and added to LB and formed a new subcategory called luminal B/HER2– (LBH–) to differentiate it from the luminal B/HER2+ (LBH+) tumors (13 tumors [18.6%]). Ki67 was significantly higher in LB than in luminal A \((p = 0.02)\) and in LBH– or LBH+ compared to A \((p = 0.001,\) for both comparisons). Ki67 was not significantly different between LBH– and LBH+ \((p = 0.99)\). Relapses occurred in 46.2% of LB patients compared to 28.1% of LA patients \((p = 0.15)\). When ki67 was considered in subtyping,
relapses were 38.7% in LBH− patients compared to 15.4% in LA patients and 46.2% of LBH+ patients ($p = 0.08$).

**Disease free survival (DFS)**

The median DFS was not reached. The 2, 3 and 5 year DFS rates for the whole group were 91%, 83% and 51% with a standard error of the mean (SEM) of 3.3%, 4.6% and 9.4%, respectively (Fig. 2A). DFS was significantly correlated with TNM stage ($p = 0.03$, Fig. 3A) and intensity of ER expression ($p = 0.02$, Fig. 3B). The 3- and 5- years DFS in patients with a Ki67 <14% were 96% and 76%, respectively, and in those with Ki67 ≥14% they were 77% and 46%, respectively, ($p = 0.046$, Fig. 4A). The 3- and 5- years DFS in patients with a Ki67 <30% were 97% and 67%, respectively, and in those with Ki67 ≥30% they were 70% and 49%, respectively, ($p = 0.001$, Fig. 4B).

There was no significant difference in DFS among different luminal subtypes ($p = 0.08$, Fig. 5A). For LA subtype, the 3- and 5- year DFS rates were 96% and 75%, respectively. For LBH− subtype, the 3- and 5- year DFS rates were 81% and 57%, respectively. For LBH+ subtype, the 3- and 5- year DFS rates were 70% and 0%, respectively. Also, there were no significant differences in DFS among different histologic subtypes ($p = 0.64$), grades ($p = 0.79$), T stages ($p = 0.59$), N stages ($p = 0.08$), PR expression ($p = 0.98$), HER2+ and HER2− patients ($p = 0.06$), those who received or did not receive neoadjuvant chemotherapy (NACT; $p = 0.54$), adjuvant chemotherapy (ACT; $p = 0.4$), adjuvant radiation therapy ($p = 0.2$) or BCS and MRM ($p = 0.65$).

**Overall survival (OS)**

At the last visit, 58 patients were alive and 12 were dead; 10 with evidence of relapse and 2 without such evidence. The median OS was not reached for the whole group or any subtype. The 3- and 5- year OS rates for the whole group were 98% and 77% with SEM of 1.5% and 6.2%, respectively (Fig. 2B).
The 3- and 5-year OS in patients with a Ki67 <14% were 100% and 90% and in those with Ki67 ≥14% were 98% and 70% (p = 0.14, Fig. 6A). The 3- and 5-year OS in patients with a Ki67 <30% were 100% and 93% and in those with Ki67 ≥30% were 97% and 60% (p = 0.007, Fig. 6B). There was no statistical difference in OS among different subtypes (p = 0.22, Fig. 5B). For LA subtype, the 3- and 5-year OS rates were 100% and 90%, respectively. For LBH− subtype, the 3- and 5-year OS rates were 97% and 75%, respectively. For LBH+ subtype, the 3- and 5-year OS rates were 92% and 60%, respectively. Also, there were no significant differences in OS among different histologic subtypes (p = 0.71), grades (p = 0.97), T stages (p = 0.34), N stages (p = 0.29), TNM stages (p = 0.17), among different ER or PR expression (p = 0.08 and 0.90, respectively), among HER2+ and HER2− patients (p = 0.14), those who received or did not receive NACT (p = 0.18), ACT (p = 0.18), adjuvant radiation therapy (p = 0.5), or between MRM and BCS (p = 0.68).

### Multivariate analysis

Using cox regression analysis to detect independent variables (covariates) affecting DFS and OS; variables that were significantly related to outcome in univariate analysis or had borderline significance were involved in two models. Model 1 (Ki67 <14% or ≥14%) revealed non-significance. Whereas in model 2 (Ki67 <30% or ≥30%), Ki values ≥30% raises the likelihood of recurrence about 4 times as compared to Ki67 values <30% (HR = 3.7; CI 95%, 1.4–9.6; p = 0.007). Also TNM stage III as compared to stage I & II raises the likelihood of recurrence to about 3 times (HR = 2.6; CI 95%, 0.97–7.2; p = 0.057).

### Discussion

Compared to no treatment, adjuvant tamoxifen for 5 years in hormone receptor positive reduced the annual BC recurrence
rate by 40% and death by 31%, irrespective of the use of chemotherapy, age or other tumor characteristics [9]. The data of adjuvant tamoxifen were recently announced. Compared to 5 years, 10 years of tamoxifen further reduced BC recurrence, BC mortality and all-cause mortality by 13%, 17% and 11%, respectively. By 15 years, BC recurrences decreased from 25% to 21% and BC mortality from 15% to 12% [30].

In their review article, Ring and Dowsett [10] mentioned that up to 40% of patients receiving adjuvant tamoxifen will eventually relapse and die from their disease. In the current study and after a median follow up of 53 months, 31% of the patients relapsed while receiving adjuvant tamoxifen. Distant, locoregional and contralateral relapses were documented in 30%, 5.7% and 1.4%, respectively compared to 8.6%, 2.1% and 1.1%, respectively in the BIG1-98 trial [20]. This may be explained by the higher tumor grades and the more advanced disease as evidenced by larger tumor size and more involvement of the axillary lymph nodes for patients in the current study compared to the mentioned study.

Saphner et al. [31] reported that the risk of recurrence of BC is higher in the first 5 years after diagnosis, but with the highest peak within 2–3 years of diagnosis, independent of nodal and hormone receptor status. In the current study, the earliest recurrence occurred 18 months from diagnosis and 17 months from the date of curative surgery. All patients received adjuvant tamoxifen for a median duration of 44 months and the median time from tamoxifen start to recurrence was 27 months. Todorovic-Rakovic et al. [32] found that, hyper-activated HER2 signaling network results in activation of the non-genomic pathway of ER via hyper-stimulation of the MAPK/Erk pathway that results in down-regulation of ER leading to generation of the ER-negative phenotype and finally, tamoxifen resistance. In agreement with this theory; 46% of the patients who were positive for HER2 in the current study developed recurrence despite receiving adjuvant tamoxifen. This could be explained by the combined effects of HER2 overexpression, high Ki67 and the complete absence of treatment with anti-HER2 therapy, all these factors increased the risk of relapse in this group. We showed a significant association between HER2 overexpression and Ki67 score (p = 0.02).

Thus, possible explanations for the relation between high Ki67 score and the development of tamoxifen resistance can be due to increased expression of HER2-associated genes and a cell proliferation signature that includes the expression of MKI67, CCNB1, and MYBL2, which has been associated with tamoxifen resistance in the studies done by Marcom et al. [33] and Oh et al. [34].

BC is no longer considered as a single disease, subtypes can be defined by genetic array testing or approximations to this classification using IHC [35]. By gene expression profiling, Sorlie et al. [36] had identified two biologically distinct ER-positive subtypes of BC: luminal A (LA) with negativity for HER2 and luminal B (LB) with HER2 overexpression. The latter also has more proliferation and poorer prognosis than the former. Going with this classification; 81% of the patients in the current study was LA and 19% was LB. The latter had worse prognosis as 46% of this group developed relapse compared to 28% of the LA group. Similar to the results reported by Viale et al. [23] and Nishimura et al. [37], Ki67 expression in the current study was significantly higher in HER2 positive (i.e. LB) compared to HER2 negative (i.e. LA) tumors (p = 0.02).

After the results of the meta-analysis on 12155 patients done by de Azambuja et al. [18] had revealed that high Ki67 positivity conferred a higher risk of relapse and a worse survival in patients with early BC; Cheang et al. [28] further classified the luminal tumors by the use of IHC for ER, PR, HER2 and Ki67. They used a cut-off value of 14% for Ki67 to assign tumors into LA (Ki67 < 14% and HER2 negative), LB/HER2 negative (Ki67 > 14% with HER2 negativity) and LB/HER2 positive (any Ki67 and HER2 overexpression). This cut-off value was subsequently accepted by the St. Gallen 2011 Breast Cancer Conference [35]. Applying this classification to the current study; 37%, 44% and 19% of the patients were assigned to LA, LB/HER2 negative (LBH−) and LB/HER2 positive (LBH+), respectively. The latter represented ~30% of all LB patients and this is similar to what reported by Perou et al. [38] and Hu et al. [39]. Ki67 expression was statistically significantly different among these groups (p < 0.001).

Regarding prognosis for these luminal subtypes in the current study; 15%, 39% and 46% of the patients in the LA, LBH− and LBH+ developed relapse, respectively, but this
was statistically non-significant \((p = 0.08)\). We believe that if our sample was larger, this finding could have reached the level of statistical significance. These findings collectively confirm the impact of Ki67 as a proliferation marker on the biological behavior of the tumor and that Ki67 identifies a subgroup of patients originally allocated to LA and has a poor prognosis similar to LB.

In the current study, the median Ki67 value was 22.5% (IQR, 10%–50%). This is similar to the median of 20.0%–22.7% mentioned by other investigators [37,40]. However, it is higher than 12% that was mentioned by Viale et al. [41]. Despite that patients in the latter study were similar to ours – postmenopausal with a median age of 61 years—they presented with less advanced tumors (T1 in 63% vs. 19% in the current study), less nodal involvement (N0 in 58% vs. 27%), more grade I tumors (28% vs. 0%) and less HER2 over-expression (6% vs. 19%).

The best Ki67 cut-off value in the current study that predicts relapse was 30%. This is higher than most of the reported cut-off values in recent BC studies that ranged between 10% and 20% [21–23,28,37,41,42]. The higher Ki67 value that predicted relapse in our study might be due to limiting inclusion only to postmenopausal patients with luminal subtype in contrast to most of the other studies that included premenopausal as well as postmenopausal patients and were not restricted to the luminal subtype.

Ki67 in the current study was not significantly different among histologic subtypes, grades, different TNM stages. This may reflect the importance of Ki67 as an indicator of tumor biology and aggressiveness rather than tumor stage that is liable to be upgraded with a lack of awareness and underdeveloped health care systems. However, Viale et al. [23] and Nishimura et al. [37] reported contradictory findings. Sampling variability with lower grade and stage tumors could be a reason for these differences.

High Ki67 was significantly correlated with poor DFS in the current study, in agreement with what many previous reports [18,21,23,28,37,42]. These findings were also subsequently confirmed on multivariate analysis that revealed that Ki67 \(\geq 30\%\) was an independent prognostic factor raising the likelihood of recurrence about 4 times as compared to Ki67 \(< 30\%\). High Ki67 \((\geq 30\%)\) inferred worse OS compared to low \((< 14\%)\) or intermediate levels \((14\%–29\%)\) \((p = 0.007)\). These results are also similar to those reported by de Azambuja et al. [18], Guarneri et al. [42] and Nishimura et al. [37].

In the current study, Ki67 was significantly higher in patients who relapsed in \(\leq 24\) months from tamoxifen start compared to those without relapse in the same period \((p = 0.007)\). This significant finding was not found in the comparison between patients who developed relapse \(> 24\) month from tamoxifen start and those who were free of recurrence in the same period \((p = 0.2)\). This result might give a clue to that, postmenopausal patients with tumors expressing high Ki67 score may develop early resistance to tamoxifen and consequently, develop early relapse. Similar to our results; patients in BIG1–98 study with high Ki67 received tamoxifen had a hazard ratio for a DFS event that was about double that of patients who received letrozole. Despite this finding was not statistically significant \((p = 0.09)\), the authors concluded that high Ki67 might identify a group of patients that can benefit from initial adjuvant letrozole [20]. Based also on this, aromatase inhibitors might be the preferred initial adjuvant hormonal therapy in this subgroup of patients [31].

Conclusions and recommendations

Unfortunately, Egyptian postmenopausal BC patients present with an advanced disease where aggressive therapies are mandated with an overall less favorable outcome. Increasing awareness among patients and health care professionals and adoption of screening may lead to earlier diagnosis with a better outcome. Neoadjuvant therapies and adjuvant taxanes were infrequent in the current study. Health education may ensure best use of such treatment modalities. Ki67 must be incorporated in the recurrence and mortality risk models that guide adjuvant chemo and hormonal therapies. We strongly recommend the universal adoption of the IHC4 panel (ER, PR, HER2, Ki67) in the initial assessment of any BC. We believe that the incremental cost of adding Ki67 to the triple-receptor panel is small but worthy. Management of BC in the adjuvant setting should be molecular subtype-oriented e.g. chemotherapy (particularly including taxanes) and aromatase inhibitors to be offered in the adjuvant setting when dealing with luminal B postmenopausal BC patients. HER2+ patients should receive adjuvant trastuzumab. Luminal A patients are less responsive to chemotherapy and hormonal therapy in the form of tamoxifen or aromatase inhibitors may be the adjuvant treatment modality of choice.

Disclosure statement

The author denies any actual or potential conflict of interest; financial or otherwise.

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