

RESEARCH ARTICLE

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Immunohistochemical Evaluation of Ki-67 and Comparison with Clinicopathologic Factors in Breast Carcinomas

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Abstract

Background: Patients primarily received tamoxifen based on their menopausal status due to the lack of immunohistochemistry. A recent study has shown that hormonal receptors were not correlated with menopausal status, and thus, indicating that they present limited therapeutic and prognostic significance in breast cancer management. This study aimed to evaluate Ki-67 value and analyze its association with clinicopathologic parameters in breast cancer patients. **Methods:** The formalin-fixed paraffin-embedded breast tissue blocks of 125 patients with primary breast carcinomas were subjected to immunohistochemical analysis using Ventana Benchmark[®] GX automated immunostainer. Analysis of variance and Chi-2 test were used to examine the relationship between Ki-67 and clinicopathologic variables. **Results:** The mean age of 125 patients included in the study was 47.7 years. The average score of Ki-67 was 56.0%. 84.8% of patients showed Ki-67 \geq 14%. Mean scores of Ki-67 were correlated with grade ($p = 0.006$), PR ($p = 0.026$), histological type, ER, combined ER/RP, and molecular subtype ($p < 0.001$). Ki-67 was independent of HER2 ($p = 0.402$) and menopausal status ($p = 0.471$). The frequency of Ki-67 according to St Gallen 2011 was associated with histological type ($p = 0.005$), grade ($p = 0.005$), ER ($p < 0.001$), combined ER/PR ($p = 0.004$), and molecular subtype ($p = 0.004$). There was no significant relationship between the distribution of Ki-67 and the age of the patients ($p = 0.859$), menopausal status ($p = 0.979$), PR ($p = 0.149$), and HER2 ($p = 0.597$). **Conclusion:** Ki-67 is useful for treatment decisions in primary breast cancer patients. The high value of Ki-67 was associated with adverse clinicopathologic factors. The increased Ki-67 value should be carefully investigated in triple negative patients.

Keywords: ER- HER2- Ki-67- PR- primary breast cancer

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Introduction

Breast cancer is the most common cancer in women worldwide (Ferlay et al., 2012; Ferlay et al., 2013). The incidence of breast cancer is increasing in sub-Saharan Africa, where it is the most frequent leading cause of death in women (Ferlay et al., 2012; Ferlay et al., 2013). Breast tumor malignancy is the most prevalent cancer in women in Ivory Coast followed by cervical cancer according to the Cancer Registry of Abidjan (Ferlay et al., 2013). Several classic breast carcinoma markers, such as estrogen receptor (ER), progesterone receptor (PR), Ki-67 proliferative index, and human epidermal growth factor of receptor 2 (HER2) are relevant for therapy strategy and prognosis (Perou et al., 2000; Sorlie et al., 2001; Cheang et al., 2009; Cuzick et al., 2011). Ki-67 antigen, described by Gerdes (1983), is a marker of proliferation controlling the cell cycle. The Immunohistochemistry (IHC) analysis of Ki-67 is the reference test to assess

tumor cell proliferation in breast tissue embedded in paraffin according to the International Ki-67 assessment in Breast Cancer Working Group (Dowsett et al., 2011). This marker presents predictive and prognostic value in clinical management of breast cancer (Stuart-Harris et al., 2008; Fasching et al., 2011; Denkert et al., 2013). Although the IHC determination of Ki-67 is integrated into routine clinical practice, its positive cut-off level remains controversial (Cheang et al., 2009; Fasching et al., 2011; Aleskandarany et al., 2011; Goldhirsch et al. 2011; Polley et al., 2013). Prior to the advent of IHC in Ivory Coast in 2013, breast cancer patient care was primary based on chemotherapy and hormonotherapy. The eligible criterion for hormonotherapy was based on patients' menopausal status due to the lack of routine IHC testing of ER, PR, HER2, and Ki-67. Studies done by Effi et al., (2017) have shown that assumption should be considered since they clarified that RH status was not correlated with menopausal status. Hereby, the menopausal status

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and the hormonal receptor (HR) status present limited prognostic and therapeutic significance in clinical care of the breast cancer patients. Today, IHC examination of Ki-67 with ER, PR, HER2 identifies five molecular breast cancer subtypes for effective and adequate personalized therapy (Perou et al., 2000; Sorlie et al., 2001; Cheang et al., 2009). However, no studies have evaluated the prevalence, distribution and, theranostic implications of Ki-67 proliferative index in breast cancer patients. The purpose of this study was to assess the value of Ki-67 and its association with menopausal status, Nottingham grade, histological type, expression levels of ER, PR, and HER2 to guide the therapeutic choice of breast cancer patients.

Materials and Methods

Patients

The prospective study was conducted between October 2014 and June 2015, including 125 patients diagnosed with primary invasive breast carcinomas at the Central Laboratory in Abidjan, Ivory Coast. The histologic diagnosis was performed upon paraffin-embedded breast tissue blocks sampled from 111 (88.8%) core needle biopsies and 14 (11.2%) surgical samples. On each sample, the histologic type and the Nottingham grade were established according to the WHO Classification of breast tumors (Lakhani et al., 2012) and the criteria of Elston and Ellis (1991), respectively. The studied variables were classic clinicopathologic parameters (age, menopausal status, histological type, and tumor grade) and hormonal receptor status (ER, PR, combined ER/PR), Ki-67, and HER2. Paraffin-embedded blocks of breast tissue were subjected to the IHC testing.

Immunohistochemical analysis

The immunohistochemical analysis was performed on 3 μ m thickness of breast tissue sections. Tissue sections were deparaffinized and heated in the drying oven BINDER® (BINDER Company, Tuttlingen, Germany) for at least 12 h at 600 C to unmask the antigenic sites. The sections were stained using the Ventana BenchMark®GX in automatic mode (Ventana Medical Systems Inc., Tucson, AZ, USA) for the assessment of ER, PR, HER2, and Ki67. The monoclonal antibody clones, developed in rats, consisted of SP1 for the ER, 1E2 for the PR, 4B5 for the HER2, and 30-9 for the Ki-67.

Immunostaining assessment

The semi-quantitative analysis was applied to the scoring systems of ER, PR, HER2, and Ki-67. The scoring of ER and PR was based on the staining intensity (weak, moderate, intense) and the percentage of tumor cells showing a nuclear immunostaining for ER and PR (range: 0-100%). Breast tissue sections were considered positive for ER and PR if $\geq 1\%$ of tumor cells, which displayed a positive nuclear staining (Hammond et al., 2010). Ki-67 score was expressed as the percentage of the number of immunostained nuclei among the total number of nuclei of tumor cells regardless of the immunostaining intensity. The counting was performed in three randomly selected fields of the breast tissue

section at x400 magnification. When the staining was homogeneous, the counting was carried out at two peripheral and one central fields. The grading of hot spots was considered in case of heterogeneous tumor (Dowsett et al., 2011). Ki-67 score ranged from 0 to 100% and its positive cut-off level was $\geq 14\%$ according to Cheang et al., (2009). The expression of proliferation index Ki-67 is categorized into 3 groups: low (Ki-67 $\leq 15\%$), intermediate (Ki-67:16-30%), and high (Ki-67 $> 30\%$) according to the recommendations of the St Gallen International Consensus of Experts (Goldhirsch et al., 2009; Goldhirsch et al., 2011). The evaluation criteria of HER2 status were based on the intensity of cell membrane immunostaining and the percentage of membrane positive cells, giving a score range of 0-3+ (Wolff et al., 2013). HER2 negative (score 0 or 1+) was decided when no staining was observed or membrane staining in less than 10% of tumor cells or a faint partial membrane staining in more than 10% of tumor cells, HER2 equivocal (score 2+) when a weak to moderate complete membrane staining in more than 10% of tumor cells and HER2 positive (score 3+) when a strong complete membrane staining in more than 10% of tumor cells. HER2 equivocal was not evaluated in our study due to the lack of fluorescence in situ hybridization.

The IHC scoring of ER, PR, HER2, and Ki-67 was reviewed independently by two pathologists. For the purpose of this study, HER2 equivocal and IHC intensity for ER and PR were not considered.

Statistical analysis

Data were collected in an Excel database from Windows 8 (Microsoft Corporation, Redmond, WA, USA) and analysed in SPSS 20.0. The analysis of variance (ANOVA) evaluated the relationship between the mean score of Ki-67 as a continuous variable with clinicopathologic factors, including menopausal status, histological type and Nottingham grade, ER, PR, HER2, combined ER/PR, and molecular subtype. The Chi-Square Test analyzed the correlation between Ki-67 as a categorical variable with clinicopathologic parameters. A probability p-value < 0.05 was considered statistically significant. The data reported as means \pm standard deviation for ANOVA and frequencies for Chi-Square Test.

Results

Characteristics of patients

The average age of all patients was 47.7 ± 10.8 years (extremes: 29-83 years). Among the 125 patients, 59.2% were premenopausal compared to 40.8% of postmenopausal. Invasive ductal carcinoma of no specific type (IDC-NST) (80.8%) was predominant. Grade II tumors were 61.1% followed by grade III (23.0%). The positivity of ER, PR, and HER2 were 54.0%, 46.7%, and 13.7%, respectively. ER/PR+ HER2- breast carcinomas (50.3%) were prevalent followed by triple negative subtype (36.8%) and HER2+ subtype (13.2%). The patients clinicopathologic factors are shown in the Table 1.

Table 1. Clinicopathologic Characteristics in the Breast Carcinoma Patients

Variables	Number of patients N=125
Age (years)	
Mean \pm SD	47.7 \pm 10.8
Extremes	29-83
Menopausal status	
< 50 years	74
\geq 50 years	51
Histological type	
IDC-NST	101
Lobular	4
Other	20
Nottingham grade	
I	18
II	69
III	26
undetermined	12
Estrogen receptor	
Positive	66
Negative	57
Uninterpretable	2
Progesterone receptor	
Positive	57
Negative	65
Uninterpretable	3
HER2	
Positive	16
Negative	101
Equivocal	3
Uninterpretable	5
Hormonal receptor	
RO+RP+	51
RO+RP-	14
RO-RP+	5
RO-RP-	50
Ki-67	
Negative (< 14%)	19
Positive (\geq 14%)	106
Molecular subtypes	
Luminal A	14
Luminal B/HER2-	43
Luminal B/HER2+	11
Non-luminal B/ HER2+	4
Triple negative	42
Unclassifiable	11

Distribution of Ki-67 mean score and clinicopathologic parameters

The average score of Ki-67 in all patients was 56.0%. Ki-67 was positively expressed in 84.8% of patients. The average score of Ki-67 was 57.8% in premenopausal

Table 2. Relationship between Ki-67 Mean Score and Clinicopathologic Factors in 125 Patients

Variables	Total (n%)	Ki-67 mean score (% \pm SD)	p
Mean age \pm SD (years)	47.7 \pm 10.8	56.0 \pm 32.6	
Menopausal status			0.471
< 50 years	74 (59.2)	57.8 \pm 33.9	
\geq 50 years	51 (40.8)	53.5 \pm 30.8	
Histological type			<0.001*
IDC-NST	101 (80.8)	58.9 \pm 31.9	
Lobular	4 (3.2)	94.5 \pm 6.7	
Other	20 (16.0)	31.5 \pm 22.9	
Nottingham grade			0.006*
I	18 (15.9)	40.3 \pm 31.5	
II	69 (61.1)	56.58 \pm 33.8	
III	26 (23.0)	71.2 \pm 21.4	
Estrogen receptor			<0.001*
Positive	66 (54.0)	45.1 \pm 31.8	
Negative	57 (46.3)	68.7 \pm 28.4	
Progesterone receptor			0.026*
Positif	57 (46.7)	48.9 \pm 32.1	
Négatif	65 (53.3)	62.0 \pm 31.9	
HER2			0.402
Positive	16 (13.7)	50.5 \pm 23.5	
Negative	101 (86.3)	57.7 \pm 32.9	
Ki-67			<0.001*
Low	19 (15.2%)	5 \pm 3.8	
Intermediate	18 (14.4%)	24.5 \pm 5.0	
High	88 (70.4%)	73.5 \pm 20.6	
Hormonal receptor			<0.001*
ER+PR+	51 (42.5)	46.3 \pm 31.5	
ER+PR-	14 (11.7)	37.2 \pm 31.3	
ER-PR+	5 (4.2)	82.6 \pm 11.1	
ER-PR-	50 (41.7)	68.2 \pm 28.8	
Molecular subtypes			<0.001*
Luminal A	14 (12.3)	6 \pm 3.8	
Luminal B/HER2-	43 (37.7)	64.4 \pm 26.4	
Luminal B/HER2+	11 (9.7)	53.3 \pm 25.6	
Non luminal B/HER2+	4 (3.5)	51.3 \pm 13.1	
Triple negative	42 (36.8)	70.6 \pm 27.8	

SD, standard deviation; p, probability p-value; (*), statistically significant difference with $p < 0.05$.

patients compared to 53.5% in post-menopausal women. There was a significant difference between the rates of Ki-67 and the histological type. Ki-67 mean score was 40.3%, 56.58, and 71.2% in Nottingham grade I, II, III, respectively. The ER+ (45.1%), PR+ (48.9%), and ER/PR+ (47.1%) patients had similarities as well as ER- (68.7%), PR- (62.0%), ER/PR- (68.2%) individuals. HER2+ patients had Ki-67 average score of 50.5% compared with 57.7% of HER2- women. The average rate of Ki-67 (70.6%) was the highest in the triple negative women while it was the lowest in the luminal A group (6%) (Table 2). The average score of Ki-67 is significantly correlated with histological type, Nottingham grade,

Table 3. Distribution of Frequency of Ki-67 According to St Gallen with Clinicopathologic Factors

Variables	Expression of Ki-67 according to St Gallen			p
	≤ 15%	16-30%	>30%	
Mean age ± SD (years)	48.9 ± 13.2	47.2 ± 11.6	47.7 ± 10.2	0.859
Menopausal status				0.979
< 50 years	11 (57.9)	11 (61.1)	52 (59.1)	
≥ 50 years	8 (42.1)	7 (38.9)	36 (40.9)	
Histological type				0.005*
IDC-NST	14 (73.7)	10 (55.6)	77 (87.5)	
Lobular and Other	5 (26.3)	8 (44.4)	11 (12.5)	
Grade				0.005*
I	5 (33.3)	5 (31.3)	8 (9.8)	
II	10 (67.0)	10 (62.5)	49 (59.8)	
III	0 (0.0)	1 (6.3)	25 (30.5)	
Estrogen receptor				<0.0001*
Positive	16 (88.9)	13 (72.2)	37 (42.5)	
Negative	2 (11.1)	5 (27.8)	50 (57.5)	
Progesterone receptor				0.149
Positive	13 (65.0)	9 (50.0)	35 (41.2)	
Negative	7 (35.0)	9 (50.0)	50 (57.5)	
HER2 status				0.597
Positive	1 (6.3)	2 (11.8)	13 (15.5)	
Negative	15 (93.7)	15 (88.2)	71 (84.5)	
Hormonal receptor				0.004*
ER+PR+	12 (66.7)	9 (52.9)	30 (35.3)	
ER+PR-	4 (22.2)	4 (23.5)	6 (7.1)	
ER-PR+	0 (0.0)	0 (0)	5 (5.9)	
ER-PR-	2 (11.1)	4 (23.5)	44 (51.8)	
Molecular subtype				0.004*
RO/RP+ HER2-	14 (87.4)	11 (68.8)	32 (39.0)	
HER2 positive	1 (6.3)	1 (6.2)	13 (15.9)	
Triple negative	1 (6.3)	4 (25.0)	37 (45.1)	

ER/PR+HER2-, luminal A and luminal B/HER2-; HER2 positive, luminal B/HER2+ and non luminal B/HER2+; SD, standard deviation; p, Probability p-value; (*), statistically significant difference with p < 0.05.

ER, PR, and ER/PR status, and molecular subtype. No correlation was found between average value of Ki-67 and menopausal status, and HER2 status (Table 2).

Distribution of Ki-67 expression according to St Gallen 2011 and clinicopathologic parameters

Premenopausal patients showed high Ki-67 (59.1%) and intermediate Ki-67 (61.1%). Ki-67 value was high in IDC-NST (87.5%). Low Ki-67 (33.3%) and intermediate Ki-67 (31.3%) were prevalent in grade I tumors. Grade II tumors were characterized by a trend of high Ki-67 in the 3 categories. Unlike grade I tumors, almost all grade III breast cancer patients had a high Ki-67, and none of them presented low Ki-67. The frequency of low and intermediate Ki-67 predominated in HR positive patients. Conversely, HR negative patients had a proportion of high Ki-67. The HER2- patients showed a less distant trend in three different groups of Ki-67, while the proportions of intermediate (11.8%) and high Ki-67 (15.5%) were

observed in HER2+ breast cancer patients (Table 3). The correlations between the frequency of Ki-67, as a categorical variable, with clinicopathologic parameters were similar to those of Ki-67 mean score, as a continuous variable, with these factors except categories of Ki-67 and PR (Tables 2 and 3).

Discussion

The evaluation of Ki-67 proliferative index is the first study done on the distribution of this marker in women with breast cancer in Ivory Coast. Interestingly, treatment of this cancer was based on ER, PR, and HER2 status after the advent of IHC in June 2013. The determination of Ki-67 and its relationship with clinicopathologic parameters have prognostic and predictive relevance (Sorlie et al., 2001; Cuzick et al., 2011; Fasching et al., 2011; Denkert et al., 2013). The average score of Ki-67 (56.0%) of the study population, whose mean age was 47.7

years, is different from that of several studies which have reported a rate varying from 20%-34.9% (Ermiah et al., 2012; Haroon et al., 2013; Ingolf et al., 2014; Wang et al., 2014; Tashima et al., 2015). The high mean score of Ki-67 in young women suggests the aggressive feature of breast cancer as shown by literature data (Haroon et al., 2013; Nishimura et al., 2014). This high value of Ki-67 could be due to the small sample size of our study population and the anti-Ki-67 antibody type. In fact, various studies have found an average score of Ki-67 which was 26.91% in 194 patients in Pakistan (Haroon et al., 2013), 29.6% in 100 patients in Libya (Ermiah et al., 2012), and 31.22% in 1,259 patients in China (Sun et al., 2015). In this current study, the IHC analysis of Ki-67 antigen used clone antibody 30-9 from Ventana in breast cancer. In contrast, other studies used a variety of antibodies such as MM1, SP6, Ki-67-S5 clones (Polley et al., 2013) and human monoclonal clone antibody MIB1 from DAKO (Dowsett et al., 2011), which is recommended. The diverse use of anti-Ki-67 antibodies in several studies could explain the significant variability of Ki-67 mean score. In addition, the evaluation of Ki-67 was carried out in the densely marked zones named hot spots in heterogeneous breast carcinomas whose number was high in our study. These hot spots were considered compared to the recommended general average score (Dowsett et al., 2011) since they have been shown to be closely associated with the survival of patients (Honma et al., 2015; Arima et al., 2016). This study identified that the expression of Ki-67, as a continuous variable, was correlated with histological type, tumor grade, molecular subtype, and ER, PR, and combined ER/PR status. However, no correlation was observed between Ki-67 as a categorical variable, and PR status, HER2 status, age, and menopausal status. The correlation of Ki-67 with clinicopathologic factors varied from one study to another, although the purpose of its investigation was similar. Objectively, Ki-67 is relevant for assessing the prognosis of patients (Stuart-Harris et al., 2008; Cuzick et al., 2011; Denkert et al., 2013), distinguishing luminal subtypes A and B (Cheang et al., 2009; Goldhirsh et al., 2011), and predicting therapeutic response (Fasching et al., 2011; Denkert et al., 2013). Undoubtedly, some authors have found relationships between Ki-67 expression and some clinicopathologic variables in a small sample size (Bouzarbar et al., 1989; Ermiah et al., 2012; Haroon et al., 2013), while others have demonstrated strong associations of Ki-67 with all clinicopathologic parameters in a large study population (Inwald et al., 2013; Park et al., 2012; Wang et al., 2014). As a result, the lack of correlation between Ki-67 and age, menopausal status, PR, and HER2 status could be due to the small size of our sample in this study. However, our study revealed that the expression level of Ki-67 is positively correlated with tumor grade. The more the tumor grade increases, the higher the Ki-67. Our result corroborates with several studies (Haroon et al., 2013; Inwald et al., 2013; Sun et al., 2015). In fact, the mitotic activity is one of the three major components of the breast carcinoma grading system according to the criteria of Elston and Ellis (1991). It reflects the intensity of cancerous cell proliferation as well as Ki-67 proliferative index which is present in different phases of

the cell cycle except quiescent phase G0 (Gerdes et al., 1984). The higher Ki-67 is associated with high grade, the more aggressive the cancer is, and thus, jeopardizing the survival of patients with early recurrence and metastasis (Haroon et al., 2013; Inwald et al., 2013). In addition, Pinder et al., (1995) have shown that grade and Ki-67 appear to be complementary rather than equivalent prognostic factors. They have found a strong correlation between Ki-67 and grade in a multivariate study. When they excluded the grade from this multivariate analysis, the positivity of Ki-67 replaced it as the most important factor in predicting the survival of patients with breast cancer. Ki-67 proliferative index and Nottingham grade clarify the aggressiveness of breast cancer, and thus, confirming their prognostic significance (Reyal et al., 2013; Inwald et al., 2013). Our research study also showed significant differences between the frequency of different scores of Ki-67 and the expression levels of ER, PR, and HER2. The positivity of ER and PR was associated with low Ki-67, whereas HER2 overexpression was observed in patients with high Ki-67. Our results are consistent with those of various researchers (Aleskandarany et al., 2011; Inwald et al., 2013; Sun et al., 2015). Our data suggests that the more aggressive the cancer is (high value of Ki-67), the lower the ER and PR expression levels or the higher the overexpressed HER2 is. Moreover, triple negative and HER2+ subtypes were associated with high score of Ki-67 compared with low Ki-67 in ER/PR+HER2- patients as confirmed by other studies (Wang et al., 2014; Sun et al., 2015). Our results indicate that triple negative and HER2+ women present worse prognosis than ER/PR+HER2- patients. A careful study should be done to investigate the high value of Ki-67 in triple negative breast cancer patients. The strong correlation of Ki-67 with molecular subtypes also suggests that Ki-67 helps predict response to neoadjuvant chemotherapy or hormone therapy in patients in our study as described by Denkert et al., (2013) and Fasching et al., (2011).

The average of Ki-67 proliferative index is high. Ki-67 is correlated with histological type, tumor grade, and ER status. However, Ki-67 is independent of age, menopausal status, and HER2 status. This study indicates that IHC testing for Ki-67 with ER, PR, and HER2, giving the breast cancer molecular subtypes, is relevant for clinical utility in patients because of its prognostic and therapeutic implications. A thorough study should be done to examine the high value of Ki-67 in young women with triple negative breast cancer.

Abbreviations

ER: Estrogen Receptor; PR: Progesterone Receptor, HER2: Human Epidermal Growth Factor Receptor 2; IHC: Immunohistochemistry; HR: Hormonal receptor; WHO: World Health Organization; ANOVA: Analysis of variance; IDC-NST: Invasive ductal carcinoma of no special type.

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Competing interests

The authors have no conflict of interest.

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