



Expression of Ki-67 and Prognosis of Breast Invasive Carcinoma in Congolese Women

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Abstract: Breast invasive carcinoma is the most cancer in the world. In low resource countries, cancer are of poor prognosis for they are diagnosed at later stage. There is not a cancer registry in the Democratic Republic of Congo and studies on biomarkers are lacking. This study had the main purpose to determine the expression of Ki-67 and the prognosis of invasive breast carcinoma in Congolese women. Cross-sectional study of 86 women with invasive Breast Carcinoma were included in the Democratic Republic of Congo from Kinshasa (n=73) and Lubumbashi Cities (n= 13). Age at the time of diagnosis, tumor size, tumor necrosis, grade of tumor and proliferation index measured by Ki-67 were taken into account. Statistical analysis used SPSS program and Pearson Chi-square test. From 2014 to 2016, biopsies were collected from 86 Congolese patients to determine the expression of Ki-67 and the prognosis of invasive breast carcinoma. The proliferation marker was observed in 91.9%. Ki-67 > 20% and > 30% were found respectively in 55.8% and in 33.7% of patients. The value of the Ki-67 was influenced by the tumor stage. The association between the size of the tumor and Ki-67 was statistically significant. The risk of tumor necrosis was 2.9 times in case of tumor with positive Ki-67. Ki-67 was positive in many patients younger than 45 years. However, the difference was not statistically significant. In patients with T3 and T4 tumors, the risk of positive Ki-67 was 7 times compared to those of T1 and T2 tumors. Patients with G3 tumors had 9 times the risk to have positive Ki-67 compared to those with G1 and G2 tumors. In conclusion, tumors in Congolese women are associated with higher proliferation index and poor prognosis for most of them are diagnosed at later stage. Chemotherapy can be justified for prior care in low resource countries and radical mastectomy should be encouraged.

Keywords: Breast Carcinoma, Prognosis, Ki-67

1. Introduction

Breast cancer is the most common cancer in the world [29]. In Africa, mortality is constantly increasing [46]. In the world, it has been shown that there are differences regarding the rate of mortality through the races [11, 13, 69]. In addition to the patients lower care access, tumors biologically are

characterized by their aggressiveness phenotype [66].

Among all the biological characteristics of tumors, cell proliferation is a cornerstone for the cancer progression [61] that explains the tumors aggressiveness and the lower survival of patients with breast carcinoma [4].

Cell proliferation is one of the important driving steps in patients with aggressive tumor phenotype and patient survival [2]. It is controlled by regulatory proteins that ensure

orderly progression of the cells through the check points of the cell cycle [60]. The cell cycle checkpoints comprise of 4 important phases which arranged sequentially: the “G1” phase prepares their machinery for duplication. The “S” phase is responsible for the genomic duplication materials; The “G2” phase is known as intervention phase, and the phase M phase controls mitosis [35]. Abnormal cell cycle regulatory protein activities are central to increase cell proliferation, poor maintenance of chromosome integrity, and therefore encouraging tumor development [65].

Many genes as CCNB1, MYBL2, and MKi-67 are implicated in proliferation. This later genes encode the Ki-67 biomarker [15, 72]. The major biological distinction between luminal A and B is the proliferation signature, including genes such as CCNB1, MKI-67, and MYBL2, which have higher expression in luminal B tumors than in luminal A tumors [41, 58].

Several biomarkers as Ki-67 are used to quantify cell proliferation [62]. Cell-cycle-associated biomarkers, such as cyclin D1, cyclin E, and p21, have been considered as prognosis factors [62]. However, the net result of cell cycling is cell proliferation and therefore immunohistochemical (IHC) analysis of Ki-67 has emerged as the marker of choice with both prognosis and treatment predictive value in breast cancer [73].

Ki-67 is one of the proliferative markers strongly linked to evaluate cell cycle [32, 78]. It was found to be universally expressed by proliferating cells (G0 and, S, G2, M) and absent in quiescent cells or G0 phase [56], making it ripe for evaluation as a tumor proliferation biomarker [77]. It represents easy and reliable method of assessing the cell cycle pathways particularly in breast cancer [19, 26, 78].

In addition to the predictive value of Ki-67 [18, 19, 42, 54, 55, 64], the prognostic significance of this protein in breast cancer of the Western women has been reported [4, 19, 21, 40, 47, 80]. Moreover, the proliferation has a major impact on calculating the risk of recurrence [27].

In Africa, data on the biology of breast cancer are lacking [6]. However, studies conducted in the USA showed that Americans of African origin have more proliferative cancers compared to Western women, and especially young women [2, 52, 57]. In England, Ganiy and Ganiyu [31] in 2012 have shown that breast carcinomas are more proliferative in patients from Africa. In fact, it is established that the biological characteristics of breast cancers depend on ethnical and racial particularities [69]. The difference in environmental factors prevents us to extrapolate the result of African women who have migrated in comparison of non-migrant African women [2, 24]. It seems possible that the biological characteristics may be different for geographic distribution [11, 70].

Nevertheless targeting Ki-67 in the indigenous black women with breast cancer might improve the prognosis in the black women with breast cancer [2]. Ki-67 biomarker provides also useful information for treatment decisions in patients with breast cancers [20, 44, 47].

The lack of study on this marker in the Democratic Republic of Congo pushes us to evaluate this in order to determine the expression of Ki-67 and the prognosis of

invasive breast carcinoma in Congolese women.

2. Materials and Method

2.1. Data Collection

Ethical approval was obtained from the research committees of our institution.

Cross-sectional study of 86 women with invasive BC was performed in the Democratic Republic of Congo from Kinshasa (n=73) and Lubumbashi (n= 13) Cities. The capital Kinshasa has approximately 7 million inhabitants and University of Kinshasa is the public institution that serves this capital. Most of sample was coming from Kinshasa University. Lubumbashi, the second town of the Democratic Republic of Congo has approximately 3 million inhabitant. Data related to the age at the time of diagnosis, tumor size, tumor necrosis, grade of tumor and proliferation index measured by Ki-67 were taken into account.

The paraffin blocks were shipped in Germany at the Martin Luther University where immunohistochemical examinations were conducted. Demographic data and tumor characteristics were obtained from medical records, and Ki-67 expression was determined using the Zytomed System. Tumor grade was assessed according to the modified Nottingham Bloom - Richardson System [25, 26]. Tumor size was described according to the TNM classification [76].

2.2. Morphological Staining

Common staining technics were carried out in the Democratic Republic of Congo and in Germany. The slides were stained according to the usual technics of haematoxylin and eosin on the basis of the following procedure:

- i) Dewaxing of slides in xylene
- ii) Rehydration in 100, 90 and 70 ° alcohol
- iii) Rinse in ordinary water
- iv) Color in the bottle of Mayer
- v) Rinse in ordinary water,
- vi) Bluing in the lithine water
- vii) Rinse in ordinary water
- viii) Color in eosin solution
- ix) Rinse in ordinary water
- x) Dehydration in alcohol of 70, 95 and 99°
- xi) Clarification in xylene

2.3. Determination of ki-67 Proliferation Index

Paraffin blocks were kept in room temperature and transferred at Martin Luther University in Germany for Immunohistochemistry.

The slides were deparaffined and rehydrated. The recombinant mouse anti-Ki-67 monoclonal antibody [clone MSK018 (Zytomed Systems GmbH Berlin, Germany); dilution, 1:2. 000 antigen retrieval with citrate buffer (PH 6.0) for 30 minutes in a hot water bath (95 c)] was used on whole sections of all breast biopsies. Secondary and tertiary immunoreactions were performed using the Dako Autostainer Plus System (Dako Cytomation, Carpinteria, CA, USA) with the anti-mouse IgG

EnVision Plus detection Kit (DakoCytomation). The diaminobenzidine was used for reaction detection according to general protocol. Sections were included in each run, which demonstrated appropriate results [1]. Three high-power fields (magnification, x40) were scored at the invasive edge of the tumors in hot spot areas. At least 100 tumor cells were counted for each section. Only nuclear staining in tumor cells and mitotic figures were assessed. The Ki-67 score was defined as the percentage of positively stained cells among the total number of malignant cells scored. Two specialized surgical pathologists independently performed the evaluation of Ki-67. In case of inconsistencies, the mean value of the two assessments was used for the final score [3]. Staining at the level less than 20% was defined as Ki-67 low or negative and those with a level more than 20% have Ki-67 high or positive [33].

2.4. Parameters Studied

Clinical data taken into account were: the age of the patient at the time of diagnosis and the size of the tumor (measured in cm).

Histological data taken into account were: visualization or not of the Ki-67 staining, the Ki-67 cut off > 20%, the histological grade (Elston and Ellis) and the stromal necrosis.

2.5. Statistical Analysis

Data analysis was performed by using SPSS statistics for windows version 19. The odd ratio and chi square tests were used to evaluate the effect of necrosis, stage at presentation and age at presentation on the probability of high Ki-67. All P values were based on two tailed tests of significance, where P less than 0.05 was considered statistically significant.

3. Results

From 2014 to 2016, biopsies were collected from 86 Congolese patients to determine the expression of Ki-67 and the prognosis of invasive breast carcinoma. The proliferation marker observed in 91.9% (Table 1). Ki-67 > 20% was positive in 55.8% (Table 2) and Ki-67 > 30% was positive in 33.7% (Table 3). The value of the Ki-67 was influenced by the tumor stage. The association between the size of the tumor and the Ki-67 was statistically significant as shown in Table 6. The risk of tumor necrosis was 2.9 times in case of tumor with Ki-67 > 20% (Table 4). Most patients younger than 45 years had tumors with a Ki-67 > 20%. However, the difference was not statistically significant as shown in Table 5. In patients with T3 and T4 tumors, the risk to score Ki-67 more than 20% was 7 times compared to those of T1 and T2 tumors (Table 6). Patients with G3 tumors had 9 times the risk to have Ki-67 more than 20% compared to those with G1 and G2 tumors as reported in Table 7.

Table 1. Expression of Ki-67 in Invasive breast (proliferation marker).

Ki-67	Number	Percentage
Absent	7	8.1
Now	79	91.9
Total	86	100

The Ki-67 proliferation marker was positive in 91.9% of the cases.

Table 2. Distribution of tumor according to the cut off of Ki-67

Ki 67	Number	Percentage
≤ 20	38	45.2
> 20	48	55.8
Total	86	100.0

According to Ki-67, 55.8% of tumors had a Ki-67 > 20%.

Table 3. Distribution of Invasive breast carcinoma according to the value of Ki-67 (low, intermediate, high).

Ki- 67	Number	Percentage
Low (≤ 15%)	32	37.2
Intermediate (16-30%)	25	29.1
High (> 30%)	29	33.7
Total	86	100.0

Tumors with a low, intermediate and a higher Ki-67 represented respectively 37.2, 29.1 and 33.7%.

Table 4. Relationship between the marker Ki-67 (proliferation) and necrosis.

Ki-67%	Necrosis		OR IC 95%	P
	Presence	Absence		
> 20	19 (39.6%)	29 (60.4%)	2.90	0.03
≤ 20	7 (18.4%)	31 (81.6%)	1, 06-7, 92	
Total	26	60		

This table shows that 39.6% of tumors with a Ki-67 > 20 had tumor necrosis and 18.4% with Ki-67 ≤ 20. Tumor with Ki-67 > 20 had 2.90 times risk to have necrosis. This association was statistically significant (p = 0, 03).

Table 5. Association between the Ki-67 and patients 'age groups.

Age	Ki-67		OR IC 95%	P
	> 20	≤ 20		
≤ 45 years	23 (60.5%)	15 (39.5%)	1.41	0.43
> 45 years	25 (52.1%)	23 (47.9%)	0.60 - 3.34	
Total	48	38		

Ki-67 was positive in 60.5% patients younger than 45 years and in 52.1% of patients of more than 45 years. The difference was not statistically significant.

Table 6. Association between the size of the tumor and the Ki-67.

Ki-67	Tumor Size		Total
	T3 and T4	T1 and T2	
>20%	43	5	48
≤20%	21	17	38
Total	64	22	86

OR= 6.96; CI = 95% (2. 26 -21. 45); Chi-square =13.1 and p< 0.001.

This table shows that patients with T3 and T4 tumors had 7 times the risk to score Ki-67 more than 20% compared to those of T1 and T2 tumors. The difference was statistically significant.

Table 7. Relationship between Ki-67 and grade of tumor.

Ki-67	Grade of Tumor		Total
	G3	G1 and G2	
>20%	37	11	48
≤20%	10	28	38
Total	47	39	86

OR= 9.4; CI= 95% (3.5- 25.3); Chi-square =22.0 and p< 0.0001

This table shows that patients with G3 tumors had 9 times the risk to score Ki-67 more than 20% compared to those of G1 and G2 tumors. The difference was statistically significant.

4. Discussion and Comment

The aim of this study was to determine the expression of Ki-67 and the prognosis of invasive breast carcinoma in Congolese women.

The Ki-67 Antigen is a molecule of nuclear localization of nature protein non histone located in the nuclear cortex [56]. It is involved in the early stages of the synthesis of ribosomal RNA by the enzyme RNA polymerase I. This protein has been identified for the first time by J. Gerdes et al, in 1983, in a cell line of a lymphoma Hodgkin [32]. The molecule was named Ki in reference to the University of Kiel, the number 67 was the number of the clone of the antibodies able to detect. The gene coding for Ki-67 (MKi-67) is located on chromosome 10 in position 10q 25 - ter and made up of 15 exons and 14 introns. Exon 13 has 16 repetitions of a pattern highly preserved 66 base pairs, called the Ki-67 pattern [22]. Ki-67 is expressed in the nucleus during G1, S, G2 and M of the cell cycle, but not during the G0 cell state of quiescence [56, 61].

The expression of Ki-67 is conventionally detected by Immunohistochemistry (IHC) in order to evaluate cell proliferation in tissue, and is reported in the form of a Ki-67 index, often simply referred as 'Ki-67' [56]. This represents the percentage of cells marked within the population studied. In cancers, it is the percentage of marked tumor cells [56].

4.1. Preanalytical Validity

As for any immunodetection, several parameters pre analytical such as setting time, the type of fixative, the duration of fixation and slides storage may interfere with the detection of Ki-67 [21]. In this study, the fixing was upon receipt of the tissue. Neutralized formalin was used and took in account the size of the mass for 2 and 48 hours. Paraffin blocks were stored in place at room temperature as recommended for pre analytical validity [51, 56]. The Immunohistochemistry examination was conducted on paraffin blocks archived for several months. It is recognized that the antigenicity can be stored on paraffin blocks even for decades [12, 14]. It is also known that the Ki-67 is one of the most robust biomarkers in immunohistochemistry. It presents relatively constant markings on tissue samples treated under different conditions such as fixation, pre-treatment conditions and immunohistochemistry protocols [10, 56, 59].

4.2. Analytical Validity

Analytical validity was done by an automated standard protocol called Zytomed systems in Germany. This included the analytical and post analytical phases.

4.3. Objectification of the Ki-67

This proliferation marker has been objectified in 91.9% in this study. It is well known that there is a positive correlation between the Ki-67 expression, the rate of cell proliferation and the active phase of the cell cycle in breast carcinoma [1, 2]. Most tumor in this study (91.9%) are in the active phase of the cell cycle or left the proliferation phase. Some authors reported a half-life of Antigen estimated at less than 90 minutes [26, 78]. In 8.1% of cases, the Ki-67 has not been highlighted. Cells at the time of the biopsy sample were in the phase of quiescence (G0) [1]. Gong et al. [34] in China, has highlighted the Ki-67 in 71, 9% in their series.

4.4. Cut off of Ki-67

The cut off of this maker of proliferation is not unanimous. It has a variability of the thresholds used to define a high Ki-67. For some authors, Ki-67 is positive from 5% to 34%, or even more [18, 56]. This variability is related to a lack of standardization of the methodology for the evaluation of Ki-67 [56, 61]. Others think that the best Cut off depends on different molecular sub-types [28]. Some researchers believed that the Ki-67 was a continuous marker, as Ki-67 reflects the percentage of proliferating cells in the tumor which can reach any value between 0 and 100% from a tumor biological point of view. And they stated that the optimal cut-off point for Ki-67 might not exist [19]. Meanwhile, transforming continuous variables into two categories can lead to a loss of power and a statistical model with continuous values could provide more information [48]. Others researchers appealed to develop accepted cut off points [79]. In this study, the Cut off of 20% was considered as proposed by the conference of St. Gall in 2013 [33]. This value is the most used in terms of prognosis [18]. So taking a cut off 20%, 55.8% of tumors had a Ki-67 > 20% in this study. For Xue C et al. [78] in China tumors with the Ki-67> 15% represented 62.1%. The study of Agboola [2] showed higher Ki-67 from Nigerian women compared with British women. This present study is another argument that ki-67 expression may be higher in black women. However, the value of Ki-67 can be influenced by time or stage of diagnosis. For Kashiwagi [45], advanced tumors may have a proliferative activity higher than tumors at the early stage. In Europe, breast carcinoma is often diagnosed at the early stage [31, 52]. In this study, the diagnosis was made more at stage T3 ouT4 and are therefore very proliferative at the time of diagnosis. These tumors are characterized by a higher proliferative status and a fast rate growth. These might contribute to their aggressiveness as reported in other areas [1, 66].

Another interest is the level of the tumor proliferative behavior. It directly influences sensitivity and cancer

treatment. The more proliferative are tumors, the more they are chemo-sensitive for most anti-cancer drugs. It is known that drugs are active on cells in one or more phases of the cell cycle [1, 8, 75]. Studies on the level of Ki-67 before neo adjuvant chemo therapy showed that tumors with a higher Ki-67 have a better chance to meet the neo-adjuvant treatment by administering the cytostatic that target rapidly dividing cells [36, 81].

Whether the tumor is hormon-sensitive or not, the Ki-67 is useful to determine the patients who may benefit from chemotherapy [44]. The great proliferation of breast carcinoma observed in this study may be due to the combination of the nature of the tumor determined by the tumor signature and the duration of its evolution. We do not prejudge the importance of one on the other.

Breast carcinomas in African patients are have been reported to be very aggressive [2, 52, 57]. Chen et al [17] reported an association between breast cancer adjusted for age, stage, socioeconomic status, body mass index, reproductive history, insurance status, and rental. They found that breast cancer in black patients are characterized by nuclear atypia and necrosis compared to white women. In a review of the literature on tumor aggressiveness in black women, Morris and Mitchell [50] report that in addition to these known differences in pathologically defined subtypes and BRCA mutations, black women also have more overexpression of cell-cycle regulators, such as cyclin E, p16, and p53, and polymorphisms in nucleotide excision repair genes. Meanwhile, recent studies on genome-wide association implicate novel breast cancer risk variants in women of African ancestry [16]. However, it is clear, that biological factors cannot explain all of the racial disparity in morbidity and mortality [49, 52].

4.5. *Ki-67 and Stage of the Disease*

Seventy four patients in this study have been received at stage III or IV. There is a statistically significant association between the tumor size of and the Ki-67. If some studies do not find association between the tumor size and the Ki-67 [34], others however attest it [2, 18, 37, 45, 67]. It is well known that during the evolution, mutations may induce cancer phenotype. These are the explanation of cancer aggressiveness and finally chemo-resistance [68]. This study shows a great number of tumors with a high level of proliferation. Denkert [19] defined groups of highly proliferating tumor as chemo or hormon-resistant. Other authors showed that tumors very proliferative and hormon-sensitive are Tamoxifen resistant [20, 48, 53]. Dumontet et al. [23] noticed that high values of Ki-67 ($\geq 32\%$) is a predictor of response to adjuvant chemotherapy. But this observation is moderated or even refuted by other especially in patients with axillary lymph node metastasis. For Agbola et al. [2], Ki-67 overexpression may be involved in the lack of response to endocrine therapy and conventional chemotherapy in Nigerian tumors. Neoadjuvant may be useful for patients in Africa in locally advanced tumors. This may provide proof if drugs can be used effectively on tumor

before surgery. The absence of multigenic signature tests in most African countries must lead to assess the ratio between the benefit and the side effects of the decision whether or not to proceed with chemotherapy or to stop it in case of chemo-resistance. The neo-adjuvant chemotherapy may be useful for a possible excision surgery of tumor into healthy margin because it induces the size of tumor to decrease [43, 68]. At the end, such decision may improve patients' overall survival and survival without recurrence as proposed by Chen et al. [18]. This study shows that the tumor size is proportional to the value of Ki-67. Only 1.1% tumors are diagnosed at stage T1. Mammography screening is not yet evaluable. For future studies, evaluation of Ki-67 in patients at the early tumor stage may be useful. It will eliminate the possible role of mutations on the tumor evolution and then on tumor proliferation.

4.6. *Ki-67 and Stromal Necrosis*

Tumor necrosis may be linked to Ki-67 value. in this study, tumor Necrosis is highlighted in 30.2% of cases. Necrosis is of poor prognosis and is the proof of a fast rate growth that exceeds the tumor stimulated angiogenesis as reported by Galant et al. [30]. This study highlights that expression of Ki-67 $> 20\%$ has 2.90 times the risk to induce necrosis. Vissler [74] found also the presence of necrosis in the high-grade cancers.

4.7. *Ki-67 and Tumor Grade*

There may be association between tumor grade and Ki-67 expression. This association has been enlightened in this study for in grade 3 tumor, KI-67 is positive in 78% of cases. Many other studies highlighted this observation [9, 34, 47, 70]. Mitotic index is one of the components to determine the grade of tumor. It is also used to evaluate proliferation. For Abadie [1], mitosis is only a small proportion of cell cycle representing morphologically the tissue in proliferation. He believes that evaluation of the proliferation only by microscopic observation, mitotic activity may lead to an underestimating of it since it does not take into account the cells in the other phases of the cycle (G1, S, G2). In Van Diest et al.'s hypothesis, evaluation of proliferation by only the number of mitoses per area may not take into account all proliferating cells [71]. In fact, the two methods are complementary even if they have different signification. The mitotic index is an estimation of the number of actual dividing cells whether Ki-67 shows the fraction of tumor cells in the dividing cycle. This later is a mean to estimate the fraction of tumor cells in continuous division and gives a reliable measure for it provides more information [63]. Apart from this correlation between tumor grade and ki-67, it is possible to make classification of grade 2 tumors tumors using the later variable [5, 38]. In fact, Heudel et al. [38] and Aleskandarany et al. [5] postulated that grade 2 tumors present a problem of reproducibility. According to them, Ki-67 may be used to divide the grade 2 tumors in two subclasses. In the present study, grade 2 tumors with Ki-67 $>$

20% represented 31.4% and have not any intermediate prognosis.

4.8. Ki-67 and Age

Data in this study show that in patients younger than 45 years, Ki-67 is positive than in those of more than 45 years. This finding is however not statistically significant. This absence of significance may be due to the small size of the sample as Awadelkarfim *et al.* [9] in 2012 supposed it. It has also been reported that cancers are more proliferating in youth [7, 34, 37, 39].

4.9. Limitations and Strengths

This study has with no doubt some limitations: the first one is the small sample size. Secondly, it is the lack of standardization as in many others studies of the cut off of the Ki-67 evaluation leading to the lack of consensus. The time of diagnosis on breast cancer in this study is later for almost all the patients. Many of them were diagnosed at T3 and T4 stage. It is possible that Ki-67 could be influenced by the stage of diagnosis in all patients. Apart of these limitations, this is a preliminary study conducted in this community. It enlightens that by using Ki-67 marker, breast cancers are very proliferative. In absence of Ki-67 marker, one can estimate the prognosis of tumors taking into account the tumor stage and standard morphology to improve cancer care in low resource countries.

5. Conclusions

The purpose of this study was to determine Ki-67 expression and the prognosis in Congolese women. Tumors are very proliferative and of poor prognosis. They are discovered at advanced stage. Chemotherapy should be used as the first care. Radical mastectomy should be considered to avoid the risk of recurrence.

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