Imperial Journal of Interdisciplinary Research (IJIR)
Vol-2, Issue-11, 2016
ISSN: 2454-1362, http://www.onlinejournal.in

Accuracy of the Duration of Tissue Fixation and the Receptor Status Profile of Primary Breast Cancers in a Tertiary Hospital of Rwanda

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Abstract*: Breast Cancer (BC) is the leading cause of cancer morbidity and mortality in women worldwide, and in Rwanda. BC receptor profile guides specific treatment; its ideal characterization requires optimal tissue fixation. While no study is yet done on BC biological profile in Rwanda, BC specimens are usually over-fixed. Objective: To determine BC receptor profiles in Rwanda and the duration of tissue fixation. All cases of invasive BC histologically diagnosed in a two-year period in a tertiary hospital in Rwanda. Optimal fixation duration was defined 24-48 hours. Blinded review was done on haematoxylin and eosin (H&E) sections for histological typing and grading, and on estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu) stained sections for receptor status. Data analysis used Stata 13.0. Two-tailed P value < 0.05 was considered significant. Forty-seven BC patients (mean age 51.7 (35-74) years; 1 male) were included. Duration of tissue fixation was optimal in 24.4% with mean duration 145.0 (24-720) hours. The main histological types were ductal (74.5%) and lobular (12.8%) among others. Most cases were of high grade (grade III in 40.4%). Molecular types were luminal A (47.8%), luminal B (15.2%), HER2+ (8.7%) and triple negative (28.3%). There was a significant association between age and BC molecular type, but not between adequacy of fixation duration and molecular type or between molecular type and histological type or grade. BC cases in Rwanda show aggressive biological behaviours. Improving the chain of transport and diagnostic infrastructure and staffing are paramount to optimize therapeutic options.*

Key words*: Breast cancer; Receptor status; Tissue fixation duration; Rwanda.*

1. Introduction

1.1 Background

Breast Cancer (BC) is the most commonly diagnosed cancer amongst women likewise worldwide (23%, or 1.38 million, of the total new cancer cases), in developed as well as developing countries; likewise it is the first cause of cancer death in women, that is, 14% of cancer deaths in women.[1] In Rwanda, BC is the second most commonly diagnosed cancer in women, with 143 cases reported clinically and/or histologically between 2000 and 2004.[2]

The incidence of BC is higher in developed countries, intermediate in South America, the Caribbean, and Northern Africa, while it is lower in Sub-Saharan Africa.[1] The geographical variations are attributed to the differences in reproductive and hormonal factors as well as the capability of early detection of the BC; but with adoption of the western lifestyle, the developing countries have tendency to

an overtime raising BC incidence.[1] Other factors of BC incidence and mortality variations include age, race/ethnicity and socioeconomic status.[3] Differences in the BC epidemiology among races may be partly attributed to endogenous hormones.[4]

1.2 Breast cancer receptor status

The growth rate of breast cancer depends on the presence of oestrogen or progesterone or both in most cases, and consequently, estrogen receptor (ER)/progesterone receptor (PR) status correlates with response to anti-hormonal therapy (tamoxifen) and chemotherapy.[5,6] ER and PR status can be determined using immunohistochemistry (IHC), and its interpretation is usually based on the Allred score semi-quantitative method.[7] Breast cancers have been divided into subtypes dependent on presence or absence of ER, PR, and human epidermal growth factor receptor 2 (HER2); luminal A tumours are (ER+, PR+) (ER−, PR+), HER−, luminal B tumours are (ER+, PR+), HER2+, HER2+ tumours are (ER−,PR−) HER2+/neu, while basal-like tumours are (ER−, PR−) HER2−, CK 5/6+ and/or CK14+ and/or EGFR1+, and unclassified tumours (ER−, PR−) HER2−, CK 5/6, CK14-, EGFR1-.[8-10] BCs that are negative for ER, PR and HER-2 (triple negative, TN) have been associated with high-grade histology, aggressive clinical behaviour, and poor survival; the same prognostic features are shared by BCs that are negative for ER and PR but positive for HER2 (double negative, DN).[11] Additionally, women with ER-/PR- first tumours had an increased risk of developing a second primary contralateral BC.[12]

The hormonal status in BC shows epidemiological disparities based on race, ethnicity, socioeconomic status, age, histological grade and stage of the tumour.[5, 8, 11, 13] Comparative studies done in developed world have showed higher proportions of ER and/or PR negative BC cases in Blacks than Whites.[11]

BC in developing countries generally shows an early peak age of onset with aggressive biological characteristics.[13] There is striking worldwide and regional variations in the proportions of BC positive to ER, PR and HER2/neu respectively.[13, 14]

1.3 Effects of fixation duration on results of receptor status in breast cancer

It is known that delay to formalin fixation has serious effect on breast biomarkers (ER, PR and HER2/neu).[15] On the matter of the fixation duration in 10% neutral buffered formalin, divergences still exist on the consequences of that duration on the receptor status results. There is a big difference in the reports regarding the optimal minimum and maximum BC tissue fixation durations.[16-18] The consensual optimal fixation duration for breast tissue for the receptor status determination is 24- 48 hours.[17]

1.4 Aims of the study

The documentation of BC hormonal receptor status and HER2/neu expression in an individual case leads to a specific treatment. In low-income countries, treatment is sometimes probabilistic, based on known preponderance of BC hormonal receptor status in a given population. In Rwanda no study on breast cancer biological profile has been yet published. This study aims at finding out BC receptor status in Rwanda as well as the duration of fixation, in the perspective of improving the good clinical and laboratory practices that guide the proper treatment of breast cancer.

2. Materials and Methods

2.1 Study area and sampling

This is a retrospective study that includes all cases of infiltrating breast carcinoma histologically diagnosed from October 2013 to September 2015 in the Department of Pathology at the University Teaching Hospital of Kigali (CHUK). The duration of fixation was determined considering the date of biopsy and the date of macroscopic examination of the specimen, as documented on laboratory request forms. Patient' s age, sex and residence were retrieved from the laboratory request form data. The type, grade, and receptor status data were retrieved from the Pathology reports in Pathology laboratory archives.

2.2 Laboratory methods

Breast tissue specimens were received fixed in 10% buffered neutral formalin. The fixation duration was considered optimal if it is 24-48 hours, short if less than 24 hours, and long if more than 48 hours. The specimens were routinely processed through the automated tissue processor according to the universal protocol for breast tissue biopsy processing. After embedding, tissue sections at 4um thickness were performed. The automated machine performed the slide staining and mounting. After histological confirmation of breast invasive carcinoma, immunohistochemistry was conducted on a selected block, through the automated immunostainer.

Archival formalin-fixed paraffin wax-embedded breast cancer tissue sections were re-assessed for grading by two blinded observers. The review of slides stained with immunoperoxidase for oestrogen receptor (ER), progesterone receptor (PR) and HER2/neu antibodies was conducted by two blinded observers. Interpretation of ER and PR status utilized the Allred score method,[7] while that of HER2/neu has used the updated guidelines from the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP).[19]

Because of the unavailability of CK 5/6, CK14 and EGFR1 antibodies in our immunohistochemistry panels, we could not distinguish the basal-like from unclassified molecular subtypes; we therefore combined both in a triple negative group. The same approach was used, though for different reasons, in a recent report from CDC (USA), in which ER and PR status were combined and analyzed as a joint HR status, and four HR/HER2 categories were used (HR+/HER2-, HR+/HER2+, HR-/HER2+, and HR-/HER2- or "triple-negative") to closely align with the four intrinsic molecular subtypes of breast cancer.[20] Likewise, we could not further characterize the cases of equivocal HER2/neu results since the fluorescence *in situ* hybridization (FISH) facility is not available in our country.

2.3 Statistical methods

Data were compiled using data collection sheets. Data entry and analysis was done through Stata 13.0 software. P value < 0.05 is considered statistically significant; all P values are two-tailed.

2.4 Ethical clearance

Ethical clearance was obtained from Hospital Research Ethics Committee. The patients' information is strictly kept confidential.

3. Results

Sixty-one biopsies from forty-seven patients were diagnosed with breast invasive carcinoma at the University Teaching Hospital of Kigali from October 2013 to September 2015, among a total number of 122 breast biopsies. The age at diagnosis varies from 35 to 74 years, with a mean of 51.69 years. Among the cancer cases, one was a 39-year-old male patient (Table 1).

41.3% of the patients were residents of Kigali City, while 26.1% came from the Eastern province. The fixation duration was optimal in 23.4% of the cases, while too long in the remaining 76.6% (Table 1). The mean fixation duration was 145.0 hours, with minimum of 24 hours and maximum of 720 hours. There was no case of short fixation duration. Invasive ductal carcinoma was the most common breast cancer histological type, representing 74.5%, followed by invasive lobular carcinoma (12.8%). Two cases of metaplastic carcinoma (4.5%) were diagnosed (Table 1). Most cases were diagnosed with high-grade tumours, with grade III in 40.4% and grade II in 51.1% of the cases (Table 1).

Table 1. Characteristics of the patients

Variables	Number	%
Age (years), *mean 51.69 years*		
≤44	12	25.5
45-49	10	21.3
50-60	13	27.7
>60	12	25.5
Gender		
Female	46	97.9
Male	1	2.1
Residence		
Eastern Province	12	26.1
Kigali City	19	41.3
Northern Province	4	8.7
Southern Province	8	17.4
Western Province	3	6.5
Not specified	1	2.1
Fixation duration, *mean 145.0 hours*		
Optimal	11	23.4
Long	36	76.6
Type of invasive breast cancer		
Ductal carcinoma	35	74.5
Lobular carcinoma	6	12.8
Medullary carcinoma	1	2.1
Metaplastic carcinoma	2	4.3
Mucinous carcinoma	2	4.3
Tubulo-lobular carcinoma	1	2.1
Cancer histological grading		
Grade I	4	8.5
Grade II	24	51.1
Grade III	19	40.4
Breast cancer receptor profile		
Luminal A	22	47.8
Luminal B	7	15.2
HER2+	4	8.7
Triple negative	13	28.3
Not done on site	1	
Total	**47**	**100**

The breast cancer receptor status profiling was performed in 46 cases, and showed luminal A in 47.8%, triple negative profile in 28.3%, luminal B in 15.2%, and HER2+ in 8.7% of cases (Table 1). There were 24 cases (51.1%) of ER+ tumours, 15 cases (31.9%) of PR+ tumours, 5 cases (10.6%) of HER2/neu+ tumours, and 6 cases (12.8%) of equivocal HER2/neu results, for which fluorescence *in situ* hybridization (FISH) was not available.

Table 2 shows the distribution of breast molecular types according to the age of the patients. The difference is age distribution among the molecular subtypes is statistically significant (P=0.015), whereby luminal A and triple negative categories occur mainly in younger patients.

The correlation analyses showed that that the mean fixation duration of breast cancer tissues was 160 hours for luminal A, 110 hours for luminal B, 155 hours for triple negative breast carcinomas and 90 hours for HER2+ tumours. There is no statistically significant difference in breast cancer molecular profiles as correlated to the adequacy of the duration of tissue fixation, as shown in table 3 (P= 0.355).

Table 2. Breast cancer molecular type and the patient's age

Molecular type	Mean age (years)	Total
A	47.54	22
B	61.57	7
Triple negative	49.46	13
HER2+	64.50	4
Total	51.69	46

Though the majority of invasive ductal and lobular carcinoma cases are in luminal A, as shown in table 4, there was no significant correlation between the type and the molecular profile of the breast invasive carcinoma (P= 0.089).

The histological grade of the breast invasive carcinoma did not correlate with the molecular type, as shown in table 5 (P= 0.532).

Table 3. Breast cancer molecular type and the duration of tissue fixation

Fixation duration	Molecular type				Total
	A	B	Triple negative	HER2+	
Optimal	3	2	3	2	10
Long	19	5	10	2	36
Total	22	7	13	4	46

Table 4. Molecular type and the type of carcinoma

Type of invasive carcinoma	Molecular type				Total
	A	B	Triple negative	HER2+	
Ductal	18	5	7	4	34
Lobular	4	0	2	0	6
Mucinous	0	2	0	0	2
Medullary	0	0	1	0	1
Metaplastic	0	0	2	0	2
Tubulolobular	0	0	1	0	1
Total	22	7	13	4	46

Table 5. Breast cancer molecular type and histological grade

Histological grade	Molecular type				Total
	A	B	Triple negative	HER2+	
I	2	0	2	0	4

II	9	6	6	2	23
III	11	1	5	2	19
Total	22	7	13	4	46

4. Discussion

Breast cancer receptor status is an important prognostic and predictive factor.[5-7, 11-13, 21] The molecular profile of the breast cancer has been categorized into five major classes, namely luminal A, luminal B, HER2+, basal-like and unclassified tumours.[8-10] In this research, we combined the basal-like and the unclassified groups into one, triple negative group. The immunohistochemistry results of breast cancer molecular profiling are controversially thought to depend on the tissue fixation duration.[16-18] However, the most accepted consensus is that the optimal fixation duration for the breast tissue destined to molecular profiling by immunohistochemistry is 24-48 hours.[17] This study aimed at determining the adequacy of breast tissue fixation and the molecular profile of breast cancer cases diagnosed in a tertiary hospital in Rwanda, in attempt to improve the good clinical and laboratory practice and set the baseline data of the breast cancer molecular types in Rwanda.

During the period of study, forty-seven patients were diagnosed with breast invasive carcinoma. The mean age at diagnosis was 51.69 years, with minimum of 35 years and maximum of 74 years. About 25.5% of the patients were below 45 years of age. In Rwanda, the BC onset is early; this is in keeping with findings in other developing countries in which BC generally shows an early peak age of onset.[13] There was no association between the residence and tissue fixation duration, meaning that long distance from home hospital to Pathology Laboratory is not the main cause of the delay to submit the breast tissue specimen in the Pathology Laboratories in Rwanda.

The fixation duration was optimal in 23.4% of the cases, while too long in the remaining 76.6%; the mean fixation duration was 145 hours, with minimum of 24 hours and maximum of 720 hours. The over-fixation of biopsy specimens in Rwanda is due to three main factors, namely the delay of specimen transport to Pathology Laboratory, the understaffing and the recurrent stock-out of reagents in the Pathology Laboratory. The resultant risk of false molecular profiles and increased turn-around time ultimately compromise the BC therapeutic approaches.[15] All stakeholders need to address these challenges in order to improve the quality of life of BC patients in Rwanda.

Invasive ductal carcinoma was the most common breast cancer, representing 74.5%, followed by invasive lobular carcinoma (12.8%). Two cases of metaplastic carcinoma (4.3%) were diagnosed. These findings are similar to those published in a recent meta-analysis study which showed that 50% to 80% of breast carcinoma cases were invasive ductal carcinoma, 5% to 11.1% were invasive lobular carcinoma, 0.2% to <5% were metaplastic carcinoma, 1.4% to 2.2% were mucinous carcinoma, 1.1% to 7% were medullary carcinoma, and up to 4.4% were pure tubular carcinoma cases.[22] Most cases were diagnosed with high-grade tumours, with grade III in 40.4% and grade II in 51.1% of the cases. As stated in previous reports, BC in developing countries generally shows an early peak age of onset with aggressive biological characteristics. [13]

The breast cancer receptor status profiling was performed in 46 cases, and showed luminal A in 47.8%, luminal B in 15.2%, HER2+ in 8.7% and triple negative in 28.3% of cases. In a study performed in Morocco, 54.3% of tumours were in luminal A, 16% in luminal B, 11.3% in HER2+ group, 11.3% in basal-like group and 7% in unclassified group.[10] These two population (Rwandans and Moroccans) groups have slightly different distributions of BC molecular subtypes; this may be explained by the geographical and racial differences. A recent study from United States of America (USA) showed that the proportion of triple negative cases (HR-/HER2-) was the highest among non-Hispanic black women.[20]

There were 24 cases (51.1%) of ER+ tumours, 15 cases (31.9%) of PR+ tumours, 5 cases (10.6%) of HER2/neu+ tumours, and 6 cases (12.8%) of equivocal HER2/neu results, for which fluorescence *in situ* hybridization (FISH) was not available. The proportions of ER+ and PR+ tumours in Sub-Saharan Africa are lower than in other areas of our planet; this explains why BCs in Sub-Saharan Africa cases are generally more aggressive.[13] The proportion of ER+ BC in Rwanda is similar to that in Nigeria (up to 65%), higher than proportions reported in Kenya (24%) and Tanzania (33%); likewise for PR+ in Rwanda BC cases, it was similar to that of Nigeria (25-56%), and higher than that reported in Tanzania (18%).[13, 14] While HER2/neu was negative in 68% of Cuban cases,[13] it was also negative in 64.2% in Rwanda cases, keeping in mind that some of equivocal cases could also turn to be negative.

The BC receptor status was was significantly associated with the age of the patient; the mean age for patients was 47.54 years in liminal A, 61.57 years in luminal B, 49.46 years in triple negative, and 64.50 years in HER2+ subtypes. These findings are similar to other reports, in which triple negative tumours were seen mainly in younger patients, with more aggressive clinical behaviours.[22]

There was no significant association between breast cancer molecular profiles and the adequacy of the duration of tissue fixation. There has been controversy in a number of reports, concerning the effect of the tissue fixation duration and the immunohistochemistry results in breast carcinoma,[16-18] with a high tendency to adopt the hypothesis that the optimal fixation duration is 24-48 hours.[17] The real effect of tissue over-fixation on immunohistochemistry results could not be assessed in this retrospective study; we therefore recommend conducting a prospective, controlled study comparing molecular profile results to a number of ranges of tissue fixation duration in Rwanda.

The type and the molecular profile of the breast invasive carcinoma were not associated, though the majority of invasive ductal luminal A, and both metaplastic carcinoma are triple negative. Large sample studies showed also that cases of invasive ductal carcinoma are predominantly of luminal A or B types, lobular carcinoma are mainly of HER2+ type, and that metaplastic carcinoma are typically triple negative.[22] The lack of association between histological type and molecular type in our series may be due to the small sample size; conducting a large multicentre prospective study will shed light on this issue.

The histological grade and the molecular type of the breast invasive carcinoma did not correlate. Reports from large cohort studies showed that BCs that are negative for ER, PR and HER-2 (triple negative, TN) or those BCs that are negative for ER and PR but positive for HER2 (double negative, DN) have been associated with high-grade histology, aggressive clinical behaviours, poor survival and increased risk of developing a second primary contralateral BC.[11-13] Larger, multicentre study comparing the histological grades and the molecular profiles and their predictive and prognostic values in BC in Rwanda is needed.

In conclusion, BC cases in Rwanda show aggressive biological (histological and molecular) behaviours, which need to be correlated to clinical behaviours in the subsequent studies. There is a long delay in submitting BC tissue specimens to the Pathology laboratory. Larger study is recommended to determine the real effect of tissue over-fixation on BC molecular profiling results. The improvement of the chain of transport and diagnostic infrastructure, supplies and staffing are paramount in guiding and optimizing the therapeutic options of BC in Rwanda.

Source of support: None

Competing interests: None to declare.

Authors' contributions: BR conceived the idea; BR, M-CN, and I-AI analysed the patients' data regarding the disease; BR, M-CN, I-AI, JDB, JBS, A-YN, NN and VB performed the histological and immunohistochemical examination of the tissue slides; BR, M-CN and I-AI did literature search, wrote the article and edited it; VB critically reviewed the article for its final content.

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