Original Research Article

SUB-CLASSIFICATION OF TRIPLE-NEGATIVE BREAST CANCER USING ANDROGEN RECEPTOR AND CYTOKERATIN 5/6

Abstract

Background

Triple negative breast cancer (TNBC) is a unique heterogenous subtypes of breast cancer which is characterized by negative estrogen, progesterone, and human epidermal growth factor receptor (HER-2) status. TNBC displays different molecular phenotype with which basal-like tumour can be identified using high molecular weight basal cytokeratin 5/6 (CK5/6).

Method

Ninety-five (95) formalin fixed cases from Korle Bu Teaching Hospital in Ghana's (KBTH) archives were sampled in a retrospective study from 2012-2016. Blocks of these triple-negative breast cancer was subclassified using CK5/6 and Androgen Receptor (AR) antibodies. Subclasses were also identified.

Results and conclusion

In all ninety-five (95) TNBC cases, hormonal subtyping was sub-classified using CK 5/6 and AR. The mean ±SD of these cases was recorded as 53.96 (±13.56) years and the age range of these cases was 22-104 years. The average size (±SD) of the tumour was recorded to be 14.43(±7.62**)** and it had a range of 2.4-45cm. lymph nodes retrieved also had a mean ± SD of 10.35(±6.05) with an average tumour lymph nodes involvement of 2.6(± 3.697).

Invasive Ductal carcinoma was identified as the commonest histologic type of TNBC with approximately 95% of the cases. This was followed by invasive lobular (2.1%), medullary carcinoma (2.1%) and metaplastic carcinoma (1.1%).

Approximately 30% of TNBC stained positive for CK5/6.

It can however be concluded that, most TNBC are not basal-like when the basal marker CK5/6 is used.

Keywords

Triple negative breast cancer

Cytokeratin 5/6

Androgen receptor

Basal-like tumour

Background

Triple-negative breast cancer (TNBC) is a heterogeneous group of breast cancers that have been confirmed with molecular profiling of the observed clinical behaviour [1]. This unique heterogeneous subtype of breast cancer is characterized by negative estrogen, progesterone and human epidermal growth factor receptor (HER 2) status. This subtype accounts for 12-20% of all breast cancers and have characteristic aggressive natural history and poor survival compared to other subtypes of breast cancers [1, 2].

Histologically, most TNBC has been shown to be invasive ductal carcinomas, characterized by high histologic grade, poor differentiation, central necrosis, high lymphocytic infiltration and high proliferative rates [2, 3]. Other several high – grade histologic subtypes of breast cancer, like medullary carcinoma, metaplastic carcinoma, adenoid cystic carcinoma, and apocrine carcinoma, also present with TNBC phenotype. There is a misconception that all triple negatives are basal-like although several types of researches have shown that not all triple negatives are basal-like (BL). The misconception continues as researchers still refer to triple-negative as basal-like [4-6]. It has been shown that TNBC display two molecular phenotypes; the basal-like TNBC and the non-basal-like TNBC (normal TNBC). This basal-like tumour can be identified using high molecular weight basal cytokeratin 5/6 (CK 5/6), CK 17, epidermal growth factor receptor (EGFR), CK14, laminin, vimentin, crystalline fascin, integrin b4, cavolin 1/2(CAV ½), P. calevin and C-Kit. It has been shown that 75-80% of TNBC display BL phenotype [1].

Aside from the use of molecular markers to distinguish between the triple-negative and basal-like, there has been substantial interest in identifying a novel therapeutic option using androgens and androgen receptor (AR) as the potential biomarker. Although there have been inconsistencies in the prognostic value of AR positivity, there is some evidence to support the role of AR in triple-negative breast cancers [7, 8]. With the availability of

androgen inhibitors e.g. bicalutamide undergoing phase II clinical trial for metastatic ER-/AR+ breast cancers, the study of prognostic value and investigation of AR as a potential target for treatment has become crucial [7, 9-12]. A study by Lehmann & Pietenpol (2014) has further identified subtypes of TNBC using gene expression and sequencing tools [13]. In that study, six subtypes were identified.

These subtypes are basal-like 1 and basal-like 2 (BL 1 and BL 2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM) and last but not the least, luminal androgen receptor [14] which is sensitive to androgen receptor antagonist [13]. TNBC has frequently been termed as basal-like (BL) molecular phenotype, although these two are not synonymous in our study site (Ghana). It is therefore important to differentiate between TNBC and BL phenotype. The gene expression method is currently not applicable to large clinical and formalin-fixed paraffin-embedded tissue and therefore immunohistochemistry has been used as an alternative in identifying basal/myoepithelial cell proteins [6].

In Ghana, no study has been done to find the various subtypes of triple-negative breast cancers. We, therefore, used Cytokeratin CK 5/6 (a basal marker) and Androgen Receptor AR (hormonal marker) to classify TNBC into basal-like 1 (CK+/AR+) and basal-like 2 (CK+/AR-), luminal androgen receptor (CK-/AR+) and luminal (normal TNBC).

Methodology

Sampling and tissue processing

Ninety-five cases of formalin-fixed paraffin-embedded tissue blocks of triple-negative breast cancers determined with ER, PR and HER 2 were selected out from 2012 to 2016 cases from the Pathology Department of Korle-Bu Teaching hospital, Ghana. This is Ghana's premier hospital with about 1500 bed capacity.

Sections of 3µm were taken from the FFPE blocks of the various cases using the microtome and having the ribbons transferred on the silane coated slides.

The tissue was the deparaffinised using xylene, ethanol and then washed in water in the following stepwise direction.

Deparaffinization

The deparaffinization process was done to remove the paraffin wax. This was done by putting tissue in three washes of xylene for 5 minutes each. Tissue was then placed into descending grades of alcohol thus 100%, 95%, 70%, and 50% for 10miutes for two washes each. Slides were then placed in distilled water for two wash for 5 minutes each. The tissues were transferred to heat retrieval stage using digital water bath at 97^{o}C for 45min with initial pre-warming at 85°C and followed by antibody treatment in the following stepwise method.

Heat retrieval and immunohistochemistry

A dedicated water bath 1.5L of distilled water and warm it to a pre-boiling temperature of 97^{o}C was used. Slides were placed in a pre-warmed staining dish containing the ImmunoDNA retrieval in the steamer, covered and steamed for 45 minutes. After heat treatment, slides were transferred in ImmunoDNA retriever with citrate to room temperature for 20 minutes and washed with changes of IHC wash buffer. Slides were placed in PolyDetector Peroxidase Blocker for 5 minutes. Tissue was covered with Primary Antibody using prediluted antibodies from BioSB (CK 5/6 and AR) for 60 minutes. Wash with 3 changes of IHC buffer. Tissue was then covered with PolyDetector Plus Link, incubated for 15 minutes and washed with 3 changes of IHC buffer. Tissue was covered with PolyDetector HRP label, incubate for 15 minutes and washed with 3 changes of IHC wash buffer. DAB was prepared by adding PolyDetector DAB Chromogen per ml of PolyDetector DAB Buffer and mixed. Tissue was covered with prepared DAB substrate-chromogen solution, incubate for 5 minutes. Rinse with 3 changes of IHC wash buffer. Counterstain Meyer's haematoxylin was used and then dehydrated and coverslip. The slides were dehydrated, cleared and mounted using the following stepwise method.

Dehydration and mounting of slides

Tissue (slides) were dehydrated in increasing order of alcohol thus two wash of 95% alcohol for 10 minutes each and also in 100% alcohol for two wash for 10minutes each. Slides were then placed in three wash of xylene for 5 minutes each. Slides were mounted with DPX and coverslip.

Reporting of the slides

CK 5/6 was said to be positive for a tumour when there was a strong or weak cytoplasmic staining and those with absent staining were considered negative for CK 5/6. However, a commercially prepared BioSB slides were used as a batch control slide which also helped in distinguishing between positive and negative CK 5/6 slides.

The slides were reported using the Allred scoring system.

Data analysis

SPSS version 25 was used for data compilation and analysis. Frequencies and percentages were calculated for quantitative variables. Mean and standard deviations were calculated for quantitative variables. Chi-square was applied to determine associations. Student t-test was applied to compare the differences in means between groups. A p- value of ≤ 0.05 was statistically significant

Results

The mean age of TNBC is 53.96±13.56 years with an average tumour size of 14.43±7.62. The highest number of cases occurred in age 50-59 with the frequency of 29 cases followed by 40-49(23 cases), 60-69(16 cases), 30-39(13 cases), 70-79 (8 cases), 80yrs (4 cases) and 20-29(1 case), as shown in Table 1.

Invasive Carcinoma NOS was the commonest histologic type with a frequency of 90 cases (94.7%) followed by invasive lobular 2 cases (2.1%), medullary carcinoma 2 cases (2.1%) and metaplastic carcinoma 1 cases (1.1%) as seen in **Table 1.**

Fifteen cases were below or equal to 40yrs while 79 cases were greater than 40yrs. Similarly, 57 cases (60.0%) occurred in age greater than 50year. This can be inferred that most of our TNBC occur in the menopausal age bracket. Fifty-three percent (53%) of the cases were in the right breast while forty-four percent (44%) were in the left breast. In terms of tumour size, 12 cases (12.6%) of the tumours were less than 5cm while 83cases (87.4%) were greater or equal to 5cm.

Most of the tumours were a high grade (Grade2 and Grade3). Grade 3 tumours accounted for 40 cases (42%), grade 2, 34 cases (35.8%) and grade 1, 5 cases (5.3%).

The mitotic activity of the tumour with less than or equal to 10 mitotic figures accounting for 28.8%, mitoses of 11-20 accounts for 38.5% and greater than 20, 32.7%.

Twenty-eight cases out of the 95 TNBC stained positively for CK5/6 (29.5%) while 70.5% were negative for the same marker. This indicates that most of the TNBC are not basal-like using the basal marker CK5/6 as shown in **table 2.**

The staining pattern of AR with 18 cases (18.9%) positivity while 77 cases (81.1%) were negative.

A combined classification of TNBC (**table 2**) was done using the CK5/6 and AR results. Seven of the cases (7.4%) were basal-like 1(positive for both CK5/6 and AR), 21 cases (22.1%) for basal-like 2 (CK5/6 positive and AR-negative), 11 cases (11.5%) were AR-positive (CK5/6 negative and AR-positive) and 56 (58.9%) cases were luminal (normal-type) (both CK5/6 and AR are negative).

Fifty percent (50%) of the TNBC show stage III disease while 44.7% show stage II disease as shown in **table 3.**

There was significance between tumour grade and CK5/6 &AR (p=0.018) and also between tumour stage and AR (p=0.014). Micrograph 1 however shows the positive results for CK5/6 and AR (E) on immunohistochemistry

Discussion

Triple-negative breast cancer is a heterogeneous group of breast cancers that have been confirmed with molecular profiling of the observed clinical behaviour[1]. This unique heterogeneous subtype of breast cancer is characterized by negative estrogen, progesterone and human epidermal growth factor receptor (HER 2) status. This subtype accounts for 12 – 20% of all breast cancers and has characteristic aggressive natural history and poor survival compared to other subtypes of breast cancers [1, 2].

Histologically, most TNBC has been shown to be invasive carcinomas NOS, characterized by high histologic grade, poor differentiation, central necrosis, high lymphocytic infiltration and high proliferative rates [2, 3]. Other several high-grade histologic subtypes of breast cancer, like medullary carcinoma, metaplastic carcinoma, adenoid cystic carcinoma, and apocrine carcinoma also present with TNBC phenotype.

There is a misconception that all triple negatives are basal-like although several types of research have shown that not all triple negatives are basal-like. The misconception continues as people still refer to triple-negative as basal-like [4-6].

In our study, the mean age for TNBC at diagnosis is 53.96 (±13.5596) which is higher than work done by Stark *et al, 2010.* which reported 48years in 75 cases of Ghanaians with TNBC [15]. In the same study, they reported 60years for African Americans and 62.4years for white Americans. Our figure is higher than what was reported for Ghanaians but lower than African-American and White American. This mean age is in line with Dent *et al, 2007.*[2].

The commonest histologic type of TNBC in this study is invasive carcinoma forming 90% as seen in similar studies [16, 17]. This histologic subtype has a poor prognostic factor making most of our TNBC poor prognostically in terms of the histology of the tumour.

More than 90% of the cases were grade II and III. Grading is an important prognostic indicator showing how differentiated the tumour resembles its normal architecture. This finding shows that there is little morphological similarity in appearance between the normal breast tissue and TNBC. Most of the TNBC is poor prognostically with reference to grading as seen in Rakha *et al, 2006.* [18].

Fifty percent of the TNBC were stage III and 47% is stage II disease giving TNBC bad prognosis in relation to the staging. This shows the extensive nature of the disease thereby reducing the five-year survival of patients with TNBC in Ghana.

Basal breast cancer has been defined by the expression molecules as tumours that display basal cluster genes that include CK5/6, EGFR, and C-KIT low expression of HER 2 neu, proliferative Cluster and hormonal related genes [17, 19-21]. In this way, however, TNBC has been classified into two basal-like (BL 1 and BL2), mesenchymal, mesenchymal stem-like, immunomodulated and luminal androgen subtypes [17], [22] using gene expressivity.

In our current study, we used two antibodies CK5/6 and AR to classify TNBC into 4 subtypes that mimic the six classes of genes expression classification. Although subtyping TNBC is not currently recommended by ASCO/CAP for clinical management, it helps to predict the prognosis of the subtypes of TNBC.

In our study, 29.5% of the TNBC expresses CK5/6 which is a basal marker. CK5/6 has been shown to be expressed by 24 to 72% of TNBC by other studies [23,24]. This marker has shown to be a good prognostic indicator in TNBC lymph node-negative tumours [17]. Unfortunately, our cases show a mean of 2.6 ±3.699 lymph node involvement. This lymph node involvement will worsen the prognosis of the tumour since it is an important factor in prognostication compared to tumour negative lymph node CK5/6 positive tumours.

Androgen receptor has also been shown to be an important prognostic factor in disease-free survival in TNBC. The expressivity of AR in TNBC has been shown to range from 9% to 56% [25-29]. In our study, nineteen percent (19%) of the TNBC expresses AR as seen in Kayahan *et al 2014* and other published work [25-29].

There is an association between AR and tumour stage (p=0.014). This may be due to advancing tumour stage leads to loss of hormonal receptor expressivity.

Conclusion

In conclusion, most triple negative breast cancers do not express basal markers like CK5&6 and hormonal receptor AR and are therefore luminal type triple negative breast cancers as opposed to basal-like TNBC.

Ethical Approval and consent to participate

Ethical approval was obtained from the Cape Coast Teaching Hospital institutional review board before the commencement of the work. Ethical approval number was CCTHERC/EC/2019/089

Availability of supporting data.

The dataset used and analysed during this study are available from the corresponding author on reasonable request.

LIST OF TABLES

Table 1: Tumour characteristics and histologic type

Variable	N	minimum	maximum	Mean±STD

Raw age	94	22.0	104.0	53.96±13.56
Size of tumour	95	2.4	45.0	14.43±7.62
Number of LN retrieve	84	0.0	40.0	10.35±6.05
Number of LN involve	84	0.0	14.0	2.60±3.70

Age	**Frequency**	**Percentage (%)**
20-29	1	1.1
30-39	13	13.8
40-49	23	24.5
50-59	29	30.9
60-69	16	17.0
70-79	8	8.5
≥80	4	4.3

Histologic type	**Frequency**	**Percentage (%)**
Invasive Ductal Carcinoma (NOS)	90	94.7
Invasive Lobular Carcinoma	2	2.1
Medullary Carcinoma	2	2.1
Metaplastic Carcinoma	1	1.1
Total	**95**	**100**

Table 2: Tumour expression of CK5/6 and AR

Variable	**Frequency**	**Percentage (%)**

Positive	28	29.5
Negative	67	70.5
Total	**95**	**100**
Case Distribution of Positivity and Negativity for AR		
Variable	**Frequency**	**Percentage (%)**
Positive	18	18.9
Negative	77	81.1
Total	**95**	**100**
Classification of TNBC using Combination of CK5/6/AR		
Variable	**Frequency**	**Percentage (%)**
A-Basal-like 1	7	7.4
B-Basal-like 2	21	22.1
C-Androgen positive	11	11.6
D-Luminal type	56	58.9
Total	**95**	**100**

Table 3: Staging of tumour

Tumour Stage			Frequency	Valid Percent	Stages
	T1N0Mx		3	4.1	**5.5** Stage 1
	R1N0Mx		1	1.4	
	T1N1Mx		1	1.4	**25.7%** Stage IIA
	T2N0Mx		16	21.6	
	R1N1Mx		2	2.7	
	T2N1Mx		4	5.4	**19%** Stage IIB
	T3N0Mx		9	12.2	
	R2N1Mx		1	1.4	
	T1N2Mx		2	2.7	**21.7%** Stage IIIA
	T2N2Mx		4	5.4	
	T3N1Mx		6	8.1	
	T3NxMx		1	1.4	
	T3N2Mx		3	4.1	
	T4N0Mx		1	1.4	**19%** Stage IIIB
	T4N1Mx		6	8.1	
	T4N2Mx		2	2.7	
	T4NxMx		5	6.8	
	T3N3Mx		2	2.7	**9.5%** Stage IIIC
	T4N3Mx		2	2.7	
	T2N3Mx		3	4.1	
	Total		**74**	**100**	

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