Original Research Article

INTRINSIC MOLECULAR SUBTYPING OF BREAST CANCER IN LOW RESOURCE SETTING.

ABSTRACT

Background

Immunohistochemistry is an invaluable technique used clinically in the characterisation of breast cancer in various intrinsic subtypes. Such characterisation into the intrinsic subtypes is of great prognostic value in the management of breast cancer.

Methodology

Two hundred and seventy-six cases of formalin-fixed paraffin-embedded (FFPE) tissue blocks were selected from 2012 – 2016 cases from Korle Bu Teaching Hospital (KBTH). The hormonal markers Estrogen Receptor (ER), Progesterone Receptor (PR), HER 2 and Ki67 were determined for cases using a semi-automated immunohistochemical method with commercially prepared antibodies from BioSB.

Results

The commonest intrinsic molecular subtype is luminal type A (42.2%), luminal B (12.3%), Her 2+ (10.5) and TNBC (35.0%). There is a significant association between tumour size and all the intrinsic subtypes ($P < 0.05$). The luminal type A and B were associated with size <5cm while TNBC was associated with size ≥5cm. Ki67 was unfavourable for 65.5% of the cases with 21.8% favourable and 12.7% being borderline. The various subtypes are significantly associated with vascular invasion.

Discussion and conclusion

This study has shown that a greater percentage of breast cancer among Ghanaian patients are hormonal positive and should have done well on hormonal treatment but did not because of the late presentation and tumour characteristics. The study confirmed previous results of the higher incidence of TNBC in African women as compared to other ethnic groups.

Keywords: Immunohistochemistry, Intrinsic molecular subtyping (IMS), Breast cancer

INTRODUCTION

Immunohistochemistry is a valuable technique used clinically in the characterisation of breast cancers into various intrinsic subtypes. These include Luminal A (Estrogen Receptor positive (ER+) and/Progesterone Receptor positive/negative (PR±) and Human epidermal receptor negative (Her2-), Luminal B (ER+, PR+ and Her2+), Her2 (ER-, PR- and Her2+) and Triple-negative (ER-, PR- and Her2-) breast cancers. Such characterisation into these intrinsic subtypes is of great prognostic value in the management of breast cancer. Luminal A generally has good prognosis compared to the other subtypes followed by luminal B [1-3]. Her2 and Triple-negative subtypes however generally have poor prognosis [1-6]. Molecular phenotype profiling of breast cancer into these histologic subtypes has become crucial in the current era of molecular targeted therapy, and personalised treatment of breast cancer. In low resource settings plagued with financial constraints and lack of facilities, studies into the pattern of occurrence of these molecular subtypes with various demographic characteristics and the clinicopathological significance are of immense importance in setting up realistic goals of breast cancer management.

Racial differences in molecular subtypes have been reported. For example, the triple-negative subtype appears to be more common in African-American populations, especially among younger African-American women, compared with European-ancestry populations. In this work we used immunohistochemical antibodies of Estrogen, progesterone, human epidermal factor to subtype breast cancer among Ghanaian patients and correlate it with the clinicopathological features.

Materials and methods

Two hundred and seventy-seven (277) cases of formalin-fixed paraffin-embedded tissue blocks were selected out of the cases received from 2012-2016. The Estrogen Receptor, Progesterone Receptor, Human Epidermal Receptor2 and Ki 67 were determined using immunohistochemistry. 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 deparaffinised using xylene, ethanol and then washed in water followed by the immunohistochemistry process.

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 60 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. Wash with 3 changes of IHC wash buffer. Tissue was covered with Primary Antibody using prediluted antibodies from BioSB (ER, PR, HER 2 and Ki 67) for 60 minutes. (This was done separately for each of the cases in consideration).Wash with 3 changes of IHC buffer. Tissue was then covered with PolyDetector Plus Link, incubated for 15 minutes and washed with three changes of Immunohistochemistry buffer.

Tissue was covered withPolyDetector Horseradish peroxidase (HRP) label, incubate for 15 minutes and washed with 3 changes of IHC wash buffer. Diaminobenzidine (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.For each of the markers

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 Distyrene(DPX) and coverslip.

Reporting of the slides

The slides were reported using the Allred scoring system as shown in table 1 below for the oestrogen and progesterone receptors. Cases for HER 2 were reported using the algorithm Fig1.

The cases were then classified into luminal A (ER+/PR+ HER 2-), luminal B (ER+, PR±, HER 2+), HER 2+ and triple-negative (ER-, PR-, and HER 2-). For Ki 67, nuclei stain of less than 10% were said to be unfavourable, 10-20% borderline and >20% favourable.

Data analysis

Statistical Package for Social Sciences (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. P-value of ≤ 0.05 as significant.

RESULTS

In **table 1,** of the various hormonal status, luminal A, Luminal B, HER-2(+ve), and triple-negative were found to be more predominant in patients who were 40 years and above (87.9%, 81.8%, 86.2% and 84.4% respectively) whereas these hormonal status were less or not predominant in patients below the age of 40(12.1%,18.2%,13.8%,and 15.6%

respectively). There was no significant association between the hormonal status and age since p value > 0.05

In **table 2,** the various hormonal status; Luminal A, Luminal B,HER-2(+ve), Triple negative were predominate in patients with ages above 50years (61.2%, 51.5%, 55.2% and 65.6% respectively) whereas patients in their menopause with ages below 50 recorded low hormonal status(38.8%, 48.5%,44.8% and 34.4% respectively). There was no significant association between the hormonal status and menopausal status since p value > 0.05

In **table 3** Luminal A and Triple Negative significantly presented with tumour sizes above 5 cm in their widest dimension whereas tumour sizes of below 5cm were predominantly associated with Her2+ and Luminal B hormonal receptor subtypes. There was a significant association between size range of tumor and HER-2(+ve) since p value < 0.05

Table 4, shows the association between hormonal status and tumor laterality. Luminal A, Luminal B and Triple-negative all presented predominantly as bilateral lesion but there was however no statistical difference in the laterality and multiplicity of luminal A, Luminal B, HER-2(+ve), and Triple Negative. In **table 5**, the hormonal status and tumor multiplicity were compared. Luminal A hormone exhibited a higher rate of solitary of tumor (96%) and a fewer rate in multiple tumors (6%). Solitary tumors showed higher expression of Luminal B, HER-2(ve), and Triple negative (82%, 100% ,94% respectively) while multiple tumors showed less expression of these hormones (17.6%, 0%,and 3% respectively).however, there was a significant association between Luminal B hormonal status and tumor multiplicity(solitary, multiple) since p value=0.001. From **table 6**, Luminal A was predominant in invasive ductal carcinoma (NOS), invasive ductal carcinoma (mucinous), invasive ductal carcinoma (papillary), invasive lobular carcinoma, DCIS, and mixed lobular and ductal carcinoma. Luminal B is also histologically associated with IDC(nos), IDC (mucinous), and intraductal papillary carcinoma. HER-2(+ve) was histologically associated with IDC (nos), invasive lobular carcinoma,andDCIS.Triple Negative was also associated with IDC (nos), medullary carcinoma and spindle cell carcinoma. Out of 259 of Invasive ductal carcinoma (nos), luminal A was hundred and eight(108), luminal B was thirty- two (32), HER2+ was twenty-six (26) and Triple Negative was ninety-three (93). Triple-negative hormonal status was associated with unfavourable Ki67 and borderline Ki67 with none of the patients having a favourable phenotype (**Table 7**). Luminal A however predominantly presented with unfavourable Ki 67 phenotype. Age did not have any association with the Ki67 status (**Table 8**). However there was a significant association between favourable Ki67 phenotype and hormonal status since p <0.05. Also, association between unfavourable Ki67

phenotype and hormonal status was significant with p value <0.05 (**Table 7**). However, high tumour grades had the highest frequency for favourable (6), unfavourable (29) and borderline (5) Ki67 phenotype while these phenotypes had least expression in low grade tumours for favourable (4), unfavourable (2) and borderline (0) phenotypes respectively **(Table 9).Table 10** shows the presentation of hormonal status and tumour grade. With low grade tumours, Luminal A hormone was higher (83%) while luminal B and Her2+ had the least (4.2%) each. In relation to high grade tumour, Luminal A and Triple Negative had (38%) each being the highest and Her2+ had the least (11%). From **table 11**, Luminal A was predominant in patients with vascular invasion (43%) and HER-2+ was less in vascular invasion (11.4%). However, Triple Negative was higher (49.4%) in patients with no vascular invasion whereas Luminal B was less (3.9%). There was a significant association between all hormonal status and vascular invasion as p value <0.05.

In figure 1, a total of 277 patients whose hormonal status were identified using the immunohistochemistry method. There were 117(42.2%) luminal A, 34(12.3%) luminal B, 29(10.5%) Her2+ and 97(35.0%) Triple Negative. **In Figure 2**, 65.5% of patients presenting with malignant breast conditions generally presented with an unfavourable ki67. Only 21.8 % presented with a favourable Ki67 and 12.7% presented as borderline.

DISCUSSION

The mean age of presentation of breast cancer was 52.4±12.7 a value higher than some studies conducted in Africa [8] with most women presenting in the post-menopausal years of 50 years and above (54.9%).In conformity with a Galukande*et al*'s study in Uganda [8], a 2-3 fold increase in TNBC (35%) was realised when compared to studies from other Caucasian populations with a prevalence of 12-17%[9-11]. However, a 21% Triple-negative was recorded in a study in Soweto, South Africa [12]. Luminal A was the most occurring subtype representing 42.2 % compared to the Uganda study recording 38% but on the contrary to that study, the least occurring subtype was Her2+ representing 10.5% as opposed to 22%. 12.3% had Luminal B subtype as opposed to 5% in the Uganda study [8].

Triple Negatives

One of the major clinical challenges in breast cancer management is triple-negative breast cancer receptor status usually associated with poor prognosis and refractory to endocrine therapy or other available targeted therapy [13-15]. The intrinsic poor prognosis compared with other receptor subtypes stems from its high metastatic potential, high recurrence rate and poor overall survival [16].

This study recorded a high prevalence of triple-negative breast cancer associated with poorer prognosis including higher tumour grade, size and unfavourable ki67 phenotype. This further confirms the high prevalence of triple-negative breast cancer in Africans [17-19]. The study contrary to some studies [11, 20] but in keeping with others (Carey, 2006) realised triple-negative tumours to be more predominant in patients who were 40 years and above. Metastatic TNBC is associated with a high proliferation index evidenced by the high ki67 score, correlating with visceral and CNS metastases [21] and poor outcome in spite of treatment creating a major clinical challenge [16, 22]. The average survival of advanced TNBC is 12 months, much shorter than the duration of survival observed in other subtypes of advanced Breast Cancer.

In conformity with literature, this study affirmed the relative better prognosis of Luminal A tumours compared with the other subtypes. Luminal B, Her2+ and Triple-negative were all associated with higher-grade tumours whereas Luminal A was not. It was also evident in the study that luminal A and B were predominantly associated with smaller tumour sizes compared with Her2 and Triple-negative also affirming the aggressiveness and

relativelypoorer prognosis of the later in keeping with Xue*et al'*sreview in 2012. The study also identified a non-association of triple-negative tumours with vascular invasion although all other histologic subtypes had a significant vascular invasion. This may be indicative of the fact that the aggressiveness of triple-negative tumours may not be as a result of vascular invasiveness. This may explain the reason why anti-angiogenic agents such as Bevacizumab and sorafenib which areanti-VEGF on TNBC have not yielded positive outcome [23].

The identification of this receptor subtype is valuable in selecting high and low-risk subsets of patients for other personalised and targeted treatment. The high prevalence of triple-negative breast cancer in this population together with the low socioeconomic status of most patients and late reporting to health facilities is indicative of a greater challenge in breast cancer management in Ghana. The major breast cancer treatment modalities available in Ghana include Chemotherapy, radiotherapy and endocrine therapy. Limited treatment options available for patients also compounds the already worse situation. Although several promising therapeutic options are being developed for triple-negative breast cancer targeting pathways such as the Notch signalling [24], Wnt/β-catenin [25] and Hedgehog pathways [26], in addition to EGFR [27], PARP1 [28], mTOR [29], and TGF-β [29, 30] due to lack of facilities and financial constraints our patients do not benefit from either research or therapy. Early detection of cases is, therefore, a key in the management of breast cancer in Ghana since between 3-4 out of 10 patients presenting with breast cancer present with TNBC. Heightened efforts are to be made for the public to be breast aware and to ensure early reporting of cases to appropriate health facilities for prompt management. Through public health awareness creation, the general public is to be educated on the need for patients to report early to health care facilities.A national policy of screening of patients 40 years and above will be valuable in ensuring we are a step ahead of the disease as a country. Self-breast examinations and regular breast screening exercises should be encouraged. Further characterisation of tumours to elucidate the aggressiveness of tumours of African origin is highly warranted. It is also recommended that hormonal receptor status should be part of routine investigations for all histology confirmed breast cancer cases to inform the decision on the best modality of management to employ. Active inclusion of breast cancer management on the national health insurance scheme.

In conclusion,greater percentage of breast cancer among Ghanaian patients are hormonal positive and should have done well on hormonal treatment but because of the late presentation and tumour characteristics.

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.

Consent of Publication.

All authors have agreed for this work to be published.

TABLES AND FIGURES LEGEND

TABLES

Table 1: Association between Hormonal status and Age

	Age blocks			
Hormonal status	<40 years (%)	≥40 years (%)	Total	*p-value*
Luminal A	14 (12.1)	102 (87.9)	116	0.381
Luminal B	6 (18.2)	27 (81.8)	33	0.491
HER-2 (+ve)	4 (13.8)	25 (86.2)	29	0.943
Triple Negative	15 (15.6)	81 (84.4)	96	0.630
Total	**39**	**235**	**274**	

Table 2: Association between Hormonal status and menopausal status

	Age blocks			
Hormonal status	<50 years (%)	≥50 years (%)	Total	*p-value*
Luminal A	45 (38.8)	71 (61.2)	116	0.940
Luminal B	16 (48.5)	17 (51.5)	33	0.238
HER-2 (+ve)	13 (44.8)	16 (55.2)	29	0.502
Triple Negative	33 (34.4)	63 (65.6)	96	0.246
Total	**107**	**167**	**274**	

Table 3: Association between hormonal status and tumor size

	Size range of Tumor			
Hormonal status	<5cm	≥5cm	Total	*p-value*
Luminal A	11 (9.5)	105 (90.5)	116	0.210
Luminal B	4 (11.8)	30 (88.2)	34	0.128
HER-2 (+ve)	1 (3.4)	28 (96.6)	29	0.045
Triple Negative	6 (6.2)	91 (93.8)	97	0.299
Total	**22**	**254**	**276**	

Table 4: The Association between Hormonal status and tumor laterality

	Laterality				
Hormonal status	Right n(%)	Left n(%)	Bilateral n(%)	Total	*p-value*
Luminal A	60 (51.7)	54 (46.6)	2 (1.7)	116	0.279
Luminal B	18 (52.9)	15 (44.1)	1 (2.9)	34	0.398
HER-2 (+ve)	14 (48.3)	15 (51.7)	0 (0)	29	0.337
Triple Negative	55 (57.9)	39 (41.1)	1 (1.1)	95	0.145

Total	**147**	**123**	**4**	**274**

Table 5: Tumor multiplicity of the various hormonal status

	Multiplicity			
Hormonal status	Solitary n(%)	Multiple n(%)	Total	*p-value*
Luminal A	110 (94.0)	7 (6.0)	117	0.450
Luminal B	28 (82.4)	6 (17.6)	34	0.001
HER-2 (+ve)	29 (100)	0 (0)	29	0.080
Triple Negative	94 (96.9)	3 (3.1)	97	0.081
Total	**261**	**16**	**277**	

Table 6: The hormonal status distribution among the various Histologic types

	Hormonal status				
Histologic type	Luminal A	Luminal B	Her2+	Triple-Negative	**Total**
Invasive Ductal carcinoma (NOS)	108	32	26	93	**259**
Invasive ductal carcinoma (mucinous)	2	1	0	0	**3**
Invasive ductal carcinoma (Papillary)	1	0	0	0	**1**
Invasive lobular carcinoma	3	0	1	0	**4**
Medullary carcinoma	0	0	0	3	**3**
Intraductal papillary carcinoma	0	1	0	0	**1**
Spindle cell carcinoma	0	0	0	1	**1**
DCIS	2	0	2	0	**4**
Mixed Lobular and	1	0	0	0	**1**

Ductal carcinoma					
Total	**117**	**34**	**29**	**97**	**277**

Table 7: Hormonal status and Ki67 tumor cells.

	Hormonal status					
Ki-67	Luminal A n(%)	Luminal B n(%)	HER-2+ n(%)	Triple Negative n(%)	Total	*p-value*
Favourable	9 (37.5)	1 (20.0)	1 (33.3)	0 (0.0)	11	0.001
Unfovourable	10 (41.7)	4 (80.0)	2 (66.7)	20 (90.0)	36	<0.001
Borderline	5 (20.8)	0 (0.0)	0 (0.0)	2 (10.0)	7	0.128
Total	**24**	**5**	**3**	**22**	**54**	

Table 8: Association between Ki-67 and Age

	Age Block			
Ki-67	**<40 years n(%)**	**>40 years n(%)**	**Total**	***p-value***
Favourable	0 (0)	10 (21.7)	10	0.118
Unfovourable	4 (66.7)	31 (67.4)	35	0.457

Borderline	2 (33.3)	5 (10.9)	7	0.071
Total	**6**	**46**	**52**	

Table 9:Association between Ki-67 and Tumor Grade

Tumor Grade	Favourable	Unfavourable	Borderline	Total
Low grade	4	2	0	6
High grade	6	29	5	40
Total	10	31	5	46

Table 10: Hormonal status and tumour grade

Hormonal status	**Grade interpretation n(%)**		**Total**
	Low grade	High grade	
Luminal A	20(83.3)	76(37.8)	**96**
Luminal B	1(4.2)	27(13.4)	**28**
Her2+	1(4.2)	22(10.9)	**23**

Triple Negative	2(8.3)	76(37.8)	**78**
Total	**24**	**201**	**225**

Table 11: Hormonal status and vascular invasion(VI)

Hormonal status	**VIn(%) Yes**	**VIn(%) No**	**Total**	***p-value***
Luminal A	72(43.3)	29(37.7)	101	0.047
Luminal B	23(13.9)	3(3.9)	26	<0.001
Her2+	19(11.4)	7(9.1)	26	<0.001
Triple Negative	52(31.3)	38(49.4)	90	0.008
Total	**166**	**77**	**243**	

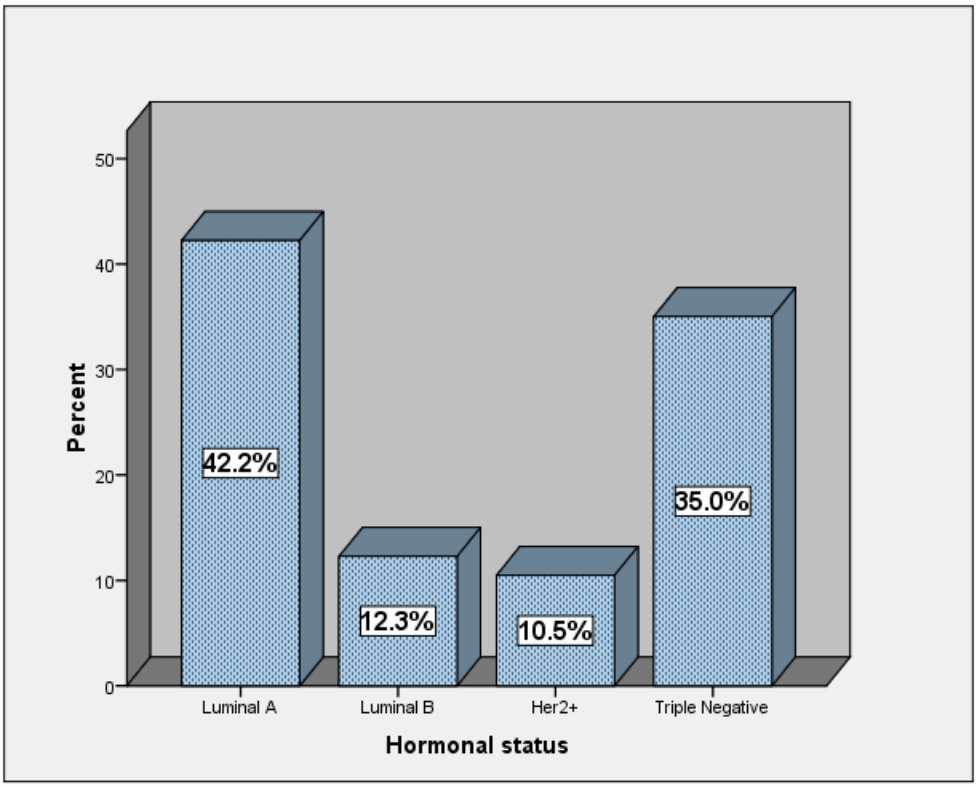

Fig 1. The hormonal status of a patient presenting with breast malignancies.

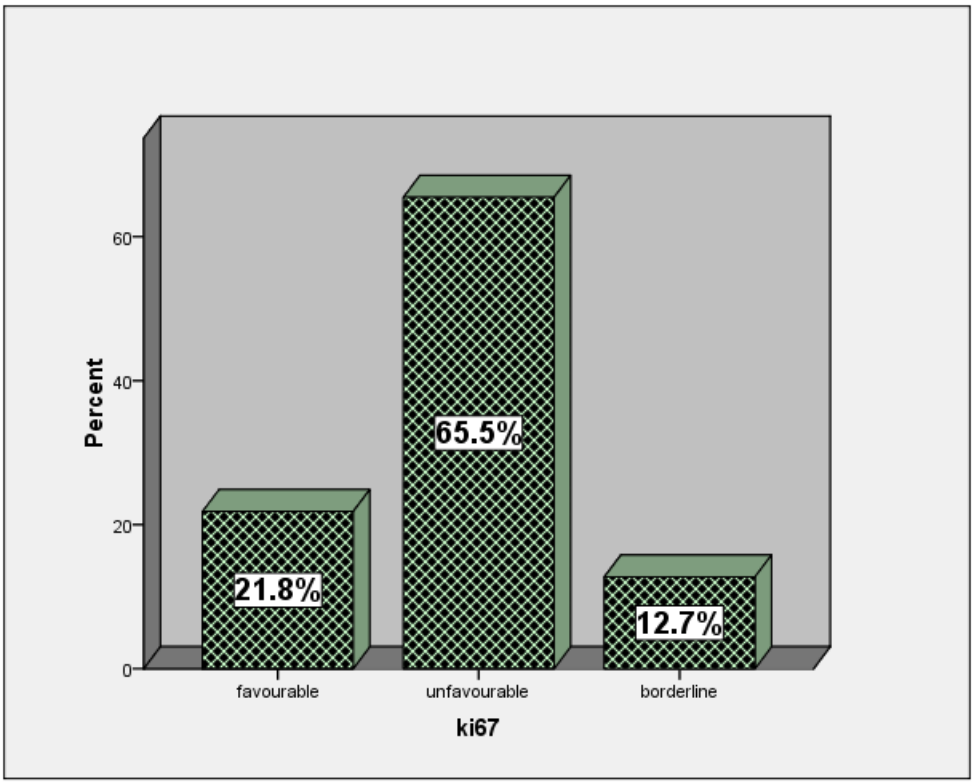

Fig 2: Pattern of distribution of Ki67 in tumour cells.

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