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RESEARCH ARTICLE

Expression of enhancer of zeste homolog 2 and cytokeratin 5/6 in triple negative versus non-triple negative breast cancer: An immunohistochemical study.

Magda Ibrahim Assaf, Essam Eldin Ismail Sabaa, Mona George Shafeek, Mona Mostafa Ahmed.
Pathology Department, Faculty of Medicine, Zagazig University, Egypt.

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Corresponding Author

Magda Ibrahim Assaf.

Abstract

Background: Triple negative breast cancer (TNBC) lacks the benefit of a specific target therapy, so identification and evaluation of new therapeutic agents is a high priority. Enhancer of zeste homolog 2 (EZH2) is a putative stem cell marker involved in cell cycle regulation and was linked to aggressive breast cancer. Cytokeratin 5/6 (CK5/6) is a basal cytokeratin used to define basal like breast cancer.

The aim: The aim of this work is to investigate the expression of enhancer of EZH2 and CK5/6 in triple negative in comparison with non-triple negative breast cancer using immunohistochemistry.

Methods: EZH2 and CK5/6 were retrospectively analyzed by immunohistochemistry in 44 paraffin-embedded specimens of breast cancer patients (20 cases of triple negative and 24 of non- triple negative breast cancer).

Results: TNBC was significantly associated with higher grade (p=0.001), high tumor budding (p=0.029), syncytial growth pattern (p=0.002), lymphovascular invasion (p=.0012), geographic necrosis (p=0.003) and lymphocytic infiltrate (p=.001). EZH2 expression is significantly associated with TNBC in comparison with non–TNBC (P=0.001). CK5/6 expression was observed in 75% of cases of TNBC in comparison to 30% of non-TNBC with a statistically significant relation between CK5/6 expression and TNBC (P=0.004). Among cases of TNBC, CK5/6 expression was significantly associated with lymph node metastasis and high tumor budding.

Conclusion: Triple negative breast cancer has distinctive but not pathognomonic morphological features. EZH2 was highly expressed in TNBC in comparison with non-TNBC and this may explain the aggressiveness of triple negative breast cancer. Basal breast cancer, identified by CK5/6 expression, showed characteristic features in the form of high tumor budding, marked lymphocytic infiltrate and higher incidence of lymph node metastasis. This finding indicates that CK5/6 positive expression in TNBC is associated with poor prognostic characteristics.

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Introduction:-

Breast cancer comprises an extraordinarily diverse group of diseases concerning morphology, molecular profile, and response to therapy**(Sørliea et al. 2001).**

Triple negative breast cancer remains the greatest challenge of all subtypes because of its clinically aggressive nature **(Yehiely et al., 2006),** increased risk of disease relapse and shortened disease-free survival **(Hussein et al., 2013).**Moreover, TNBC lacks the benefit of specific therapy that targets ER, PR and HER-2. Thus identification and evaluation of new biomarkers and therapeutic agents is a high priority (**Rakha**et al., **2007).** Enhancer of zest homologue2 (EZH2) is responsible for healthy embryonic

development through the maintenance of genes responsible for regulating development and differentiation (**Morey et al., 2010).** It was found that EZH2 has potent oncogenic properties in the breast tissue owing to its role in stem cell maintenance **(Puppe et al., 2009).**EZH2 inhibits genes responsible for suppressing tumor development (**Yoo et al., 2010).**

Basal cytokeratins refer to large number of high molecular weight cytokeratins including cytokeratin 5/6, CK14 and CK17, located in the basal cell layer (**Rakhaet al.,2007).** The expression of CK5/6 is commonly used as an immunohistochemical indicator for tumors with the basal-like gene expression profile (**Rakhaet al.,2011).**

Material and methods:-

Forty four formalin fixed, paraffin embedded tissue blocks of triple negative breast cancer (n=20) and non-triple negative breast cancer (n=24) were collected from the archives of Departments of Pathology, Zagazig Faculty of Medicine and National cancer institute, Cairo University, during the period from September 2009 to September 2013. Clinicopathological data and hormone and HER-2 status were abstracted from archive files of the corresponding departments. The specimens were obtained through both excisional biopsy (n=34) and tru-cut biopsy (n=10).

The analysis was restricted to invasive ductal carcinoma of no special type (thereby avoiding any confounding effect of special types of invasive breast cancer) and cases previously tested for ER, PR and HER-2. Cases of breast cancer with unknown ER, PR and HER-2 status, recurrent cases, cases who received pre-operative chemo or radiotherapy and cases with inadequate tissue for immunohistochemistry were excluded.

Paraffin blocks of all cases were sectioned at 3-5 micron thickness and stained with routine hematoxylin and eosin stain to evaluate the following:
1-Tumour Grading was carried out according to Nottingham Grading System**(Elston et al., 1991).** 2-Tumor budding: Peritumoral buds in ten high-power fields (HPFs) were counted according to previous reports.High tumor budding equated to scores > 4 tumor buds across 10HPFs. Tumors were considered to have low tumor budding if the average number of buds in 10 HPFs was ≤4**(Salhia et al. 2015).** Tru-cut biopsies were omitted from the study of tumor budding owing to the usual central location of the biopsy. 3- Lymph node ratio: cutoff points classified patients into low- (≤ 0.20), intermediate- (> 0.20 and ≤ 0.65), and high-risk (> 0.65) groups**(Vinh-Hung et al., 2009).** 4- Lymphocytic infiltration was evaluated as criteria of **Thike et al.** (**2010).** 5-Lympho-vascular invasion was assessed according to **Ly et al. (2012).** 6- Geographic areas of necrosis were assessed as present or absent **(Gazinska et al., 2013).** 7- Tumorgrowth pattern was evaluated as trabecular versus syncytial (**Thike et al.2010).**

Immunohistochemistry:-
Immunohistochemical staining was carried out using indirect streptoavidin-biotin immunoperoxidase technique. Tissue sections (3–5 µm) were deparaffinized in xylene and rehydrated in graded alcohol. Slides were incubated for 10 minutes in 0.3 % hydrogen peroxide in absolute methanol to block endogenous peroxidase activity. Antigen retrieval was performed using Dako target retrieval solution (pH 6.0) (Dako, CA, USA). The slides were then stained using a mouse monoclonal anti- EZH2 antibody (United States Biological – Massachusetts, USA), with a 1:100 dilution; and a ready to use mouse monoclonal anti CK5/6 antibody (Lab Vision, Corp, Fremont, CA, USA). The slides were incubated for 60 minutes at room temperature then washed with two changes of phosphate buffered saline (PBS), ph. 7.6, stained with secondary antibody for 15 minutes at room temperature, then rinsed in the buffer again. Skin and testes were taken as positive controls for CK5/6 and EZH2 respectively. Negative controls were obtained by omission of the primary antibody.

Evaluation of immunohistochemical staining:-
1- EZH2immunostaining:-
EZH2 expression is nuclear. Semi quantitative scoring of EZH2 expression was done according to following scale:
0 = negative, 1 = positivity in 1 to 5% of tumor cells (low expression), 2 = positivity in >5 to 25% (intermediate expression), 3 = positivity in >25 to 50% (high expression) and 4 = positivity in more than 50% (very high expression) (**Wagener** et **al., 2010).**A cut off point value of 25% is used to divide EZH2 expression into low (score 1&2) and high (4&3) EZH2 expressionas was formerly proposed by **Hussein et al. (2012).**

2 –CK5/6 immunostaining:-
A reaction was considered CK5/6 positive if >10% of tumor cells showed cytoplasmic and/or membranous staining **(Gazinska et al., 2013).**

Statistical analysis:-
Statistical analysis was performed using SPSS 20 for windows (2011.Armonk, NY: IBM Corp). Data was expressed as mean ±SD for quantitative variables. For categorical variables Fisher's exact test or chi-square was used. P-value less than 0.05 was considered significant.

Results:-

Among the 44 cases enrolled in this study, 20 cases were TNBC, 20 cases were hormone positive and 4 were HER2-enriched type, according to data abstracted from the files.

Statistical analysis was carried out between triple negative and non-TNBC (hormone positive) cases. The 4 HER2 / neu cases were studied separately owing to the low number found and enrolled in this study. TNBC patients were younger (48±8.7 years) than those of Non-TNBC (57.9±14.5 years). Among 4 cases of HER-2 enriched breast cancer, the median age was 52 years.

Histopathological characteristics of breast cancer cases (Table 1).

Table 1 Clinical and pathological characteristics of breast cancer cases:

Variable	Total N(40)	Triple negative breast cancer n=20(%)	Non- triple negative breast cancer (hormone positive) n=20(%)	X^2	P value
Size***					
• <2	12	6(50%)	6(50%)	.96	**0.7**
• 2-5	15	6(40%)	9(60%)		
• >5	3	2(66.7%)	1(33.3%)		
Grade					
• I	0	0(0%)	0(0%)	18.03	**0.001**
• II	16	1(6%)	15(94%)		**HS**
• III	24	19(79%)	5(21%)		
Pattern of growth					
• Syncytial	13	11(84.6%)	2(15.4%)	9.2	**0.002**
• Trabecular	27	9(33.4%)	18(66.6%)		**S**
Geographic Necrosis					
• present	15	12(80%)	3(20%)	8.6	**0.003**
• absent	25	8(32%)	17(68%)		**S**
Lymphocytic infiltrate					
• Mild	19	3 (15.8%)	16(84.2%)		
• Moderate	13	10 (77%)	3(23%)	17.16	**0.001**
• Marked	8	7 (87.5%)	1(12.5%)		**HS**
Lymphovascular invasion					
• Present	24	17(70.8%)	7(29.2%)	10.4	**0.0012**
• Absent	16	3(18.7%)	13(81.3%)		**S**
Tumor budding***				4.47	**0.029**
• High	11	8(72.7%)	3(27.3%)		**S**
• Low	19	6(31.3%)	13(68.7%)		
Lymph node metastasis***				2.62	**0.1**
• Positive	19	9(42.1%)	10(57.9%)		
• Negative	11	5(72.7%)	6(27.3%)		
Lymph node ratio***					
• Low	14	7(50%)	7(50%)	.65	**0.8**
• Intermediate	8	3(37.5%)	5(62.5%)		
• High	8	4(50%)	4(50%)		

S:Significant , HS:highlySignificant,$p< 0.05$ is significant, χ^2: Chi-square test, *** Tru cut biopsies were omitted from statistical analysis (6 triple negative breast cancer and 4 non-triple negative breast cancer)

Analysis of data presented in Table 1. revealed the following:
There was no statistically significant difference found between TNBC and **Non-TNBC** concerning size of the tumor (p=0.7).Considering histological grading, TNBC cases were predominantly high grade, in contrast to Non-TNBC. The relation was highly significant (p=0.001). The pattern of growth in TNBC cases was predominantly syncytial rather than trabecular (p= 0.002).

A statistically significant relationship was found between TNBC and geographic necrosis (p=0.003). TNBC had a prevalence of marked lymphocytic infiltrate compared with non-TNBC (p= 0.001).

Lymphovascular invasion was detected in24 of studied cases, 17 (70.8%) were TNBC and 7(29.2%) were non-TNBC. A statistically significant relationship was found between TNBC and lymphovascular invasion (p= 0.0012).

High grade tumor budding was noted in 8 cases (72.7%) of TNBC in comparison with 3 cases (27.3%) of non-TNBC. The relation was statisticaly significant (p =0.029).
Although evidence of lymph node metastasis was more common in non- TNBC (11 cases, 57.9%) than TNBC (8 cases, 42.1%), yet the difference was not statistically significant. No statistically significant difference was found between triple negative and non-triple negative breast cancer concerning lymph node ratio (p=0.8).

Histopathological data of HER2 enriched cases:-
Among the four HER-2 enriched breast cancer cases 3 belonged to grade III and one case was grade II; 3 cases revealed a trabecular growth pattern and one case showed a syncytial pattern;
Two cases showed geographic necrosis, two cases of high lymph node ratio and two revealed high tumor budding.
EZH2 expression:
EZH2 positive reaction showed nuclear expression. High EZH2 was detected in 50% of cases (20/40) (Figure 1).
Correlation of EZH2 expression with TNBC versus non- TNBC is summarized in Table 2.

Table 2 EZH2 expression in Triple negative versus non-triple negative breast cancer.

Variable	Total	EZH2		χ^2	P
		Low N=20	High N=20		
TNBC	20(50%)	4(20%)	16(80%)	14	0.001
Non-TNBC	20(50%)	16(80%)	4(20%)	.4	HS

TNBC= triple negative breast cancer
χ^2: Chi-square test, HS: highly Significant

High EZH2 expression is significantly associated with TNBC (80% of the cases) in contrast to only 20% of non-TNBC cases. (p= 0.001).

CK5/6 expression:-
CK5/6 reaction showed cytoplasmic and/or membranous staining (Figures 2&4). Correlation betweenCK5/6 expression in TNBC versus non- TNBC is presented in Table 3.

Table 3CK5/6 expression in TNBC versus non- TNBC

Score	Total	CK5/6 Negative N=19	CK5/6 Positive N=21	X^2	P
TNBC	20	5 (25%)	15 (75%)	14.5	0.004
Non- TNBC	20	14 (70%)	6 (30%)		S

TNBC= triple negative breast cancer
χ^2: Chi-square test, S:Significant

Triple negative breast cancerrevealed a more frequent positive reaction to CK5/6 (75%) in contrast to only 30% positivity in non- triple negative (hormone positive) cases. A Highly significant correlation ofCK5/6 expression with triple negative status was detected (p=0.004).

Correlation between CK5/6 expression and clinicopathological parameters of triple negative breast cancer cases (Table 4).

Table 4 Correlation between CK5/6 expression with and clinicopathological characteristic parameters in TNBC

Variables	Total	Triple negative breast cancer (n=20)		X2	P
		Ck 5/6 negative N=5	Ck5/6 positive(basal) N=15		
Size***					
• <=2	6	3(50%)	3(50%)	5.09	0.07
• 2-5	6	0	6(100%)		
• >5	2	0	2(100%)		
Grade					
• I	0	0	0	3.16	0.07**
• II	1	1(100%)	0		
• III	19	4(21%)	15(79%)		
Pattern of growth					
• Syncytial	11	3(27.2%)	8(72.8)	.07	0.79
• Trabecular	9	2(22.2%)	7(77.8%)		
Geographic necrosis					
• present	12	4(33.3%)	8(66.4%)	0.28	0.55
• Absent	8	1(12.5%)	7(87.5%)		
Lymphocytic infiltrate				7.11	
• Mild	3	3(100%)	0(0%)		0.001 HS
• Moderate	10	1(10%)	9(90%)		
• Marked	7	1(14.2%)	6 (85.7%)		
Lymphovascular invasion					
• Present	17	3(17.5%)	14(82.5%)	0.02	0.87**
• Absent	3	2(66.6%)	1(33.4%)		
Tumor budding***					
• high	8	0	8(100%)	7.06	0.007** S
• Low	6	5(83.3%)	1(16.4%)		
Lymph node metastasis ***					
• Positive	9	0	9(100%)	6.38	0.011** S
• Negative	5	3(60%)	2(40%)		
Lymph node ratio***					
• Low	7	3(42.8%)	4(57.2%)	1.7	0.19
• Intermediate	3	0	3(100%)		
• High	4	0	4(100%)		

S:Significant , HS:highly Significant, $p< 0.05$ is significant χ^2: Chi-square test, **Fisher's exact test.

*** Trucut were omitted from statistical analysis (4 cases of CK5/6 positive and 2 cases of CK5/6 negative).

Comparison between basal and non-basal TNBC showed that basal breast cancer had a significantly more lymphocytic infiltrate (p=0.001) and an increased incidence of high tumor budding (8/9) over non- basal TNBC (p=0.007).All TNBC cases with lymph node metastasis were of the basal type while none of non-basal TNBC showed lymph node metastasis (p=0.011).

Although all cases of basal breast cancer were high grade in comparison to non-basal TNBCcases (21%) , yet no statistically significant difference between the two groups was detected (p=0.07).

There was no significant difference between the two groups regarding architectural features of geographic necrosis (p=0.55) and growth pattern (p=0.79). Most of TNBC, regardless of CK5/6 expression , showed presence of syncytial growth pattern and geographic necrosis.

Lymphovascular invasion was detected in 82.5% of the cases of TN basal breast cancer (14/15) in comparison to non-basal TNBC (17.5%); the relation, however, is not significant. No significant difference was detected between basal breast cancer and non-basal TNBC concerning age, size of the tumor, and lymph node ratio.

Correlation of CK5/6 expression with EZH2 expression among triple negative breast cancer cases.
Among cases of triple negative breast cancer, high EZH2 expression was detected in 4/5 cases of the non-basal breast cancer versus 12/15 cases of basal breast cancer. By applying Pearson's correlation coefficient test, there was no correlation found between EZH2 expression and basal breast cancer (p =1).

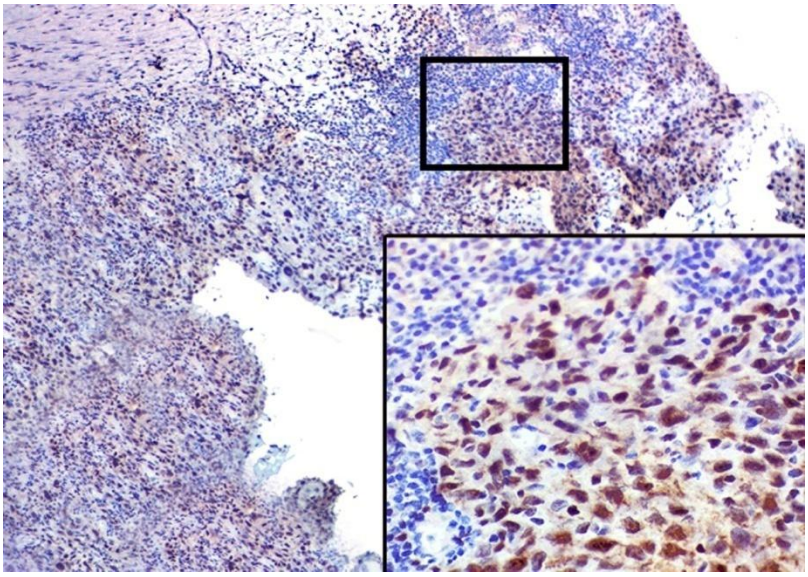

Fig. 1 High EZH2 nuclear immunohistochemical staining (>25%) in a high grade triple negative invasive ductal carcinoma, not otherwise specified (DAB, original magnification×100; Inset shows nuclear EZH2 expression X400).

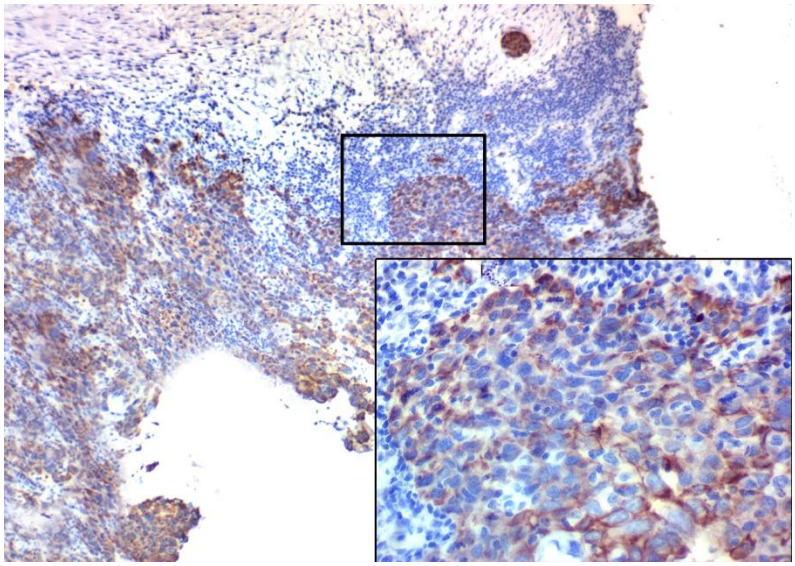

Fig 2. Same case revealing a positive CK5/6 expression (>10%) (DAB, original magnification x100; Inset shows cytoplasmic and membranous CK5/6 expression x400).

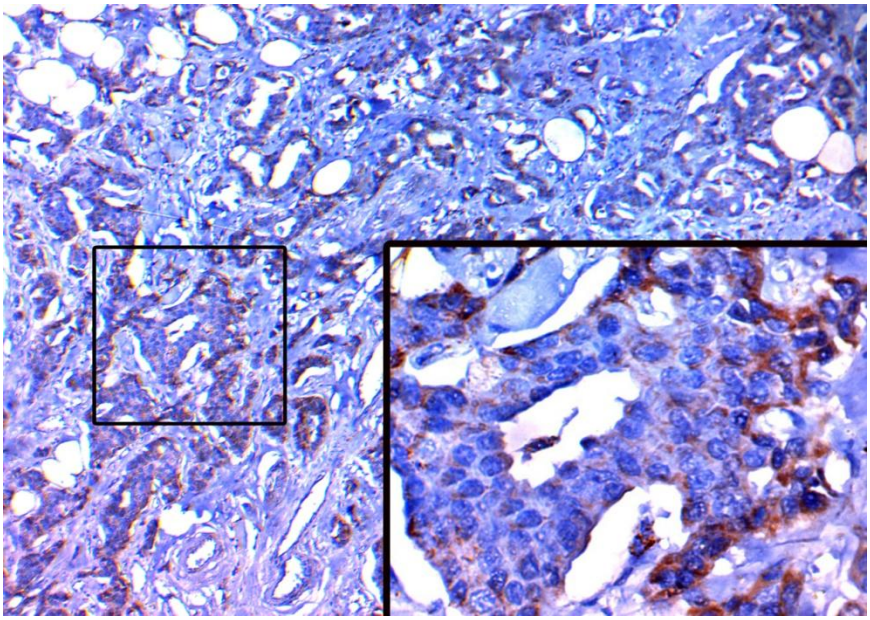

Fig. 3.Low EZH2 nuclear immunohistochemical staining (<25%) in grade II hormone positive invasive ductal carcinoma, not otherwise specified (DAB, Original magnification×100; Inset reveals negative nuclear expression of EZH2 with small foci of cytoplasmic staining X400).

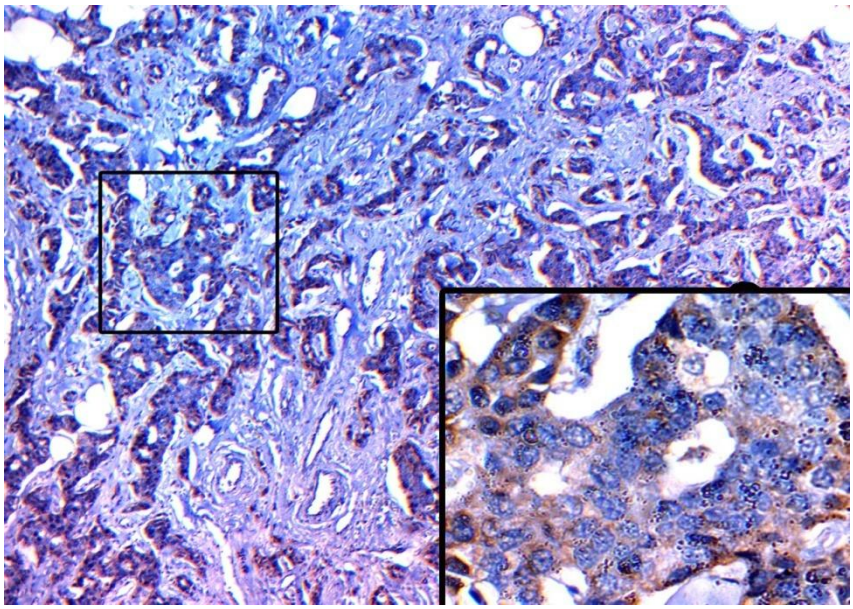

Fig.4 Same case revealing positive cytoplasmic CK5/6 expression (>10%) (DAB, original magnification×100; Inset shows cytoplasmic and membranous CK5/6 expression x400).

Discussion:-

Breast cancer is a heterogeneous group of disease with varied clinico-pathological features, clinical behavior, and various responses to therapies **(Sotiriou et al., 2009).** Heterogeneity is attributed to differences in the underlying cell of origin **(Lim et al., 2009).** Among the types of breast cancer, triple-negative breast cancers (TNBC) are clinically problematic: Unlike hormone-positive and HER-2 enriched breast cancers, TNBC lacks an approved targeted systemic therapy (Rakha et al., 2007).

Data on the incidence of breast cancer in Cairo, Egypt, as reported by the National Cancer Institute (**EL-Bolkeny et al., 2013) and Ain Shams University (Helal et al., 2015)** revealed an incidence of 20%and 23.9%, respectively. These registries, however,do not present data on the incidence of TNBC. Distinct data on the incidence of TNBC were reported in auniversity hospital in the Mansoura governorate, where the incidence of TNBC was 19% of breast cancer cases, while 66.9% of the cases were hormone receptor positive, and only 14.1% were Her-2 positive **(Hussein et al. 2013).**

According to the results of the present study, the cardinal clinico-pathological features of TNBC are different from those of non-TNBC tumours, as will be shown below.

Although the cases were randomly selected in the present study, according to strict inclusion and exclusion criteria, it is noteworthy that the triple negative status was strongly associated with the younger age group (48±8.7 years) as opposed to those of non-TNBC cases (57.9±14.5 years); an observation consistent with an earlier study by **Pillaiet al. (2012)**, who found that the majority of patients diagnosed with TNBC were younger, with a mean age of (45.3 ± 10.3) years versus (50.0 ± 10.4) years in the non-TNBC cases.This result differs from that of **Hussein et al. (2013)**, who found that the median age of the breast cancer patients was 52 years and was equal across all receptor status types. This finding needs to be further tested.

Regarding tumour size, the current study shows no significant differences between TNBC (mean size 3.5cm) and non-TNBC (mean size 4.4 cm). **Pillaiet al., (2012)** found that TNBC were larger (2.8 cm) than non-TNBC (2.5 cm) but the difference was not significant. On the contrary, **Qiu et al. (2016)**reported on a significant relation between TNBC and a tumor size more than 5 cm when compared to non-TNBC.

Applying the Nottingham system for grading in the current study has shown a predominance of grade III tumors (19/20) in TNBC cases. This is consistent with the results of a study carried out by **Rakha el al. (2009)** on 232 TNBC cases. They found that 94% of their TNBC cases belonged to Grade III. **Dent et al. (2007)** and **Pillai et al. (2012)** described similar results. In contrast, a predominance of Grade II non- TNBC (hormone-positive) cases (15/20) was found in the current study. This result is consistent with the results of**Qiu et al. (2016)**, who found that 88.3% of non-TNBC belonged to grade I& II and 11.7% belonged to grade III. These findings show the strong association between TNBC and high grade.

In the current study, two distinct patterns of growth were recognized in breast cancer specimens, namely a trabecular and a syncytial pattern. The syncytial pattern was significantly higher in TNBC cases (p=0.002), in contrast to non- TNBC where a trabecular pattern was predominating. This result is in agreement with the studies of **Salman et al.(2012) and Thike et al. (2010).**

As regards to geographic necrosis, the present study has shown its significant association with triple negative cases (p= 0.003), where 80% of the cases with geographic necrosis were TNBC. This result is in close agreement with those of **Livasy et al. (2006), Bhargava et al. (2009) and Salman et al. (2012),** who reported a percent of geographic necrosis in their series of TNBC cases of 74%, 75% and 73.5% respectively. This finding supports the view of **Foulkes et al. (2004),** who stated that geographic necrosis represents one of the characteristics of aggressive tumors.

TNBC with lymphocytic tumor infiltrate at diagnosis may benefit from immune- based therapies that are most beneficial if given in combination with cytotoxic drugs that potentiate adaptive anti-tumor immunity **(Stagg and Allard, 2013).** This assumption was put into consideration while comparing between TNBC and non- TNBC in the current study, where a highly significant association between marked lymphocytic reaction and TNBC (p= 0.001) was found, a result in agreement with the findings of **Salman et al. (2012)** who reported the presence of a marked lymphocytic infiltrate in 55.9% of TNBC and **Ono et al. (2012)** who found it in 73%. Cases of TNBC with marked lymphocytic infiltrate should be reported separately for a possible immune- based therapy

In the present study, 85% (17/20 cases) of TNBC cases revealed lymphovascular invasion. Out of the total number of specimens with vascular invasion, 70.8% were TNBC in contrast to 29.2% non-TNBC cases. The relation between TNBC and lymphovascular invasion was statistically significant (p= 0.0012). This is in agreement with the results of **Qiu et al. (2016)**, who found that 64.6% of their series of TNBC showed lymphovascular invasion. In contrast to the present results, **Mohammed et al. (2011)** described an absence of significant difference between

triple negative and non- triple negative breast cancer regarding lymphovascular invasion. One should, however, consider that high-grade malignancies, like in the case of TNBC, are expected to reveal loss of adhesion between tumor cells with a higher chance for lymphovascular invasion. Concerning tumor budding in breast cancer, budded cells display the epithelial mesenchymal transition molecular phenotype with acquisition of migratory and invasive prosperities (**Thiery et al.,2009).** That is why peritumoral budding is associatedwith high risk of loco-regional metastasis (**Salhia et al., 2015).** In the present study a statistically significant association between TNBC status and peritumoral budding was found (p = 0.029); this result further adds to the more aggressive behaviour of TNBC over non-TNBC cases.

Regarding the rate of absolute number of metastatic lymph nodes, in the current study no significant differences were observed between TNBC and non-TNBC. On the contrary,**Qiu et al. (2016),**found a significant association between TNBC and the number of metastatic lymph nodes (64.6% in TNBC versus 48.01% in non-TNBC).Also,**Pillaiet al. (2012),** found that non-TNBC show higher incidence of lymph node metastasis (44.4%) compared with 21%of TNBC but the difference was not statistically significant.Discrepancies in results could be attributed to case selection, as well as geographic and genetic differences that should be put into consideration.

According to the adopted staging system **described by Edge et al. (2010)**, the prognostic value of the absolute number of nodes removed for predicting disease burden in the axilla is confounded by the number of nodes removed. In a developing country, like Egypt, a significant number of breast cancer surgeries are performed with less than optimal axillary lymph nodes retrieval (**Elkhodary et al.,2014)**. That is why lymph node ratio (LNR) is better at predicting breast cancer specific mortality than pN staging (**Chagpar et al., 2011).** In the current study, no significant difference was found in the LNR between TNBC and non- TNBC. According to **Jayasinghe et al. (2015),**no correlation between ER status and LNR was found. There are, however, some limitations for the value of lymph node ratio evaluation in the present study: data about axillary lymph node status show wide range of number of dissected lymph nodes (5-20).

As far as we know, this is the first study that investigates the correlation between triple negative status and LNR, as well as TNBC and peritumoral budding. A cross sectional survey study should therefore be carried out to define the exact relation between these factors and TNBC.

Enhancer of zest homologe 2 (EZH2) was found to have potent oncogenic properties in the breast tissue and its oncogenic role may be ascribed to its role in stem cell maintenance (**Puppe et al., 2009).**Our findings point to a strong correlation between high EZH2 expression and TNBC phenotype, where 80% of TNBC cases revealed high EZH2 expression (p=0.001). This is consistent with **Hussein et al. (2012),** who found it in 71.9% of their TNBC cases.Different studies considered EZH2 as a stem cell marker (**Kleer, 2003, Chou et al., 2011 and Dinget al., 2014),** asserting its biological role in tumor aggressiveness and may explain why TNBC requires aggressive therapeutic regimens.

The detection of tumors with high EZH2 expression is of clinical importance as EZH2 inhibitors are now showing early signs of promise in clinical trials for the treatment of TNBC **(Kim et al., 2016).**

Cytokeratin 5/6 is an independent prognostic marker for poor prognosis of breast cancer. It is used to define basal-like breast cancer **(Kuroda et al., 2008),** and is regarded as a distinct group of tumors that is associated with a poor clinical outcome **(Rakha et al. 2008).**

Progenitor stem cells of both glandular and myoepithelial cell lineages express CK5/6 **(Ba´nkfalvi et al. 2004).** The differentiated cells do not express CK5/6 (**Bahalla et al., 2010)**. The present study demonstrated that TNBC status is strongly associated with the basal cell marker CK5/6 (p=0.04), as it was expressed in 75% of TNBC cases, which represent a basal subtype. Similar findings were reported by **Pillaiet al., (2012)**who found CK5/6 expression in 72% of their TNBC cases. According to **Salman et al. (2012),** basal tumors accounted for58.8% of triple-negative tumors. While in the study carried out by **Livasy et al. (2006),** who used a combination of CK5/6 and EGFR for identification of basal breast cancer,all TNBC cases were of the basalphenotype. According to **Tomaskovic-Crook et al (2009),** epithelial cell plasticity in breast carcinoma can generate distinct cellular subpopulations that contribute to intra-tumoral heterogeneity. However, most tumors display a dominant phenotype that enables classification of the tumor**.** This may explain CK5/6 positivity in 6 cases of hormone positive breast

cancer. To prove this further, an extended study should be conducted, utilizing the different basal phenotype markers.

Triple negative breast cancer cases were collectively associated with high grade, with no significant difference between basal breast cancer and the non-basal group. This is consistent with the finding of **Gazinska et al. (2013).**In the present study, basal breast cancer showed an increased incidence of high tumor budding than non-basal TNBC.**Sarrio et al. (2008),** found that stem cells from tumor breast tissue had a basal-like phenotype and were enriched in the expression of genes involved in epithelial-to-mesenchymal transition, resulting in increased invasive and metastatic capabilities of cancer cells. As far as we know, this is the first study that correlates CK5/6 expression with peritumoral budding.

Among cases of TNBC, lymph node metastasis was significantly associated with basal phenotype (p =0.011).This result is in agreement with that of **Joensuu et al., (2013)** (p=0.025). It is, however, different from results of **Kuroda et al. (2008) and Kim et al.**(**2006),** who found no statistically significant correlation between basal breast cancer and nodal metastasis.

A highly significant correlation was found between basal type of TNBC and the presence of marked lymphocytic infiltrate when compared with non–basal TNBC (p=0.002). This may be explained by tumoral expression of C-X-C motif chemokine 10 (CXCL10) in basal breast cancer that enhances tumoral lymphocytic infiltrate (**Mulligan et al., 2013).**

Among cases of triple negative breast cancer, no correlation was found between EZH2 expression and basal breast cancer. As far as we know, previous studies did not examine the correlation between EZH2 and basal breast cancer versus Triple negative breast cancer.

Absence of a significant difference between expression of EZH2 in triple negative basal and non-basal breast cancer, can be explained by the new classification of TNBC of **Du et al.(2015),** in which the presence of stem cells in TNBC are not restricted to the basal subtype. More extensive research should be carried out to prove this finding.

Conclusion

Triple negative breast cancer has distinctive but not pathognomonic morphological features in comparison with non-triple negative breast cancer. EZH2 which is a putative stem cell marker was highly expressed in TNBC in comparison with non-TNBC and this may explain the aggressiveness of triple negative breast cancer. TNBC of basal phenotype, as detected by CK5/6, was found to significantly associated withcharacteristic pathological features, namely high tumor budding, marked lymphocytic infiltrate and higher incidence of lymph node metastasis. Therefore reporting of basal subtype in cases of TNBC is recommended for its predictive and possibly therapeutic value.

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