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Resistance to Trastuzumab in Clinical Her2 Breast Cancer: Possible Relation to Molecular Subtype and Cancer Stem Cells

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ABSTRACT: ***Molecular breast cancer (BC) subtypes and cancer stem cells (CSCs) can determine sensitivity to trastuzumab. A better predictivevalue of clinical human epidermal growth factor receptor2 (cHER2) BC can be provided, by incorporating CSCs heterogeneityinto clinical decisions. The study aimed at investigating involvement of transition of CSCs biology and the molecular subtype indevelopment of trastuzumab resistance in HER2 BC, recommending the use of trastuzumab combinations/modifications able tosuppress CSCs and overcome trastuzumab resistance. The study was conducted on 40 BC patients age range 27-63 years, 20served as control group and 20 as metastatic (predicted resistance) group. Clinicopathological analysis included HER2, ER andPR status, chemotherapy regimen and number of herceptin cycles. Histological analysis included, molecular subtype markers(HER2 and cytokeratin (CK) 5/6), CSCs marker CD44 and morphometric studies. Quantitative polymerase chain reaction (qPCR)was performed. In the metastatic group significant decrease in the area% of HER2 positive (+ve) immunoexpression (IE),significant increase in the area% of CK5/6 IE and CD44 +ve cells was detected. qPCR values of HER2 gene confirmed asignificant decrease. It was concluded that resistance to trastuzumab in the metastatic group of HER2 BC is related to basal likesubtype and overexpression of CD44 +ve CSCs. This nictitates IHC assessment of molecular subtype and type of CSCs to allowthe choice of most appropriate treatment regimen giving best expected prognosis.***

Keywords: HER2 BC, cancer stem cells, cytokeratin, CD44, basal-likeHER2,

INTRODUCTION

Breast cancer (BC) is a highly heterogeneous disease, histopathological classification includes 20 major types and 18 minor subtypes with minimal prognostic & predictive implications **(1).** Biological classification includes expression of hormone receptors (HR), highly endocrine responsive, or human epidermal growth factor receptor (HER2) BC, not endocrine responsive (treated by trastuzumab). Molecular classification represents clustering analysis of gene expression profiling. Luminal HER2-enriched and basal-like classes were proved by molecular classification and were found to have distinctive biological and clinical features. Prognostic value is confirmed **(2).**

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The HER2 gene is amplified in 25–30% of BCs and this amplification causes overexpression of the encoded protein in 95% of cases. HER2 BC is a more aggressive phenotype and adverse disease prognosis was recorded. 1st-line treatment of HER2 +ve BC achieved a response rate (RR) of 30%–35%, a median progression-free survival (PFS) of 5–6 months and a median overall survival (OS) of 20–23 months. Addition of trastuzumab, a humanised monoclonal antibody with specificity for the HER2 protein, resulted in RR of 50%–72%, median PFS of 11-12 months and median OS of 25–36 months. It is administered as intravenous (IV) infusion, in part with chemotherapy or subcutaneous (SC) which is well tolerated with fewer adverse events **(3).**

Adjuvant trastuzumab has become foundation of care for HER2 early BC. Neoadjuvant therapy (NAT) is a successful approach to convert patients inoperable at diagnosis to operable/ make conserving surgery possible instead of mastectomy. Although trastuzumab is an effective treatment in early stage HER2 BC, the majority of advanced HER2 BCs develop trastuzumab resistance. Continued use of trastuzumab may increase the frequency of cancer stem cells (CSCs) and metastasis potential **(4)**.

Based on the spectrum of molecular BC subtypes and the CSC-determined sensitivity to trastuzumab, a better delineation of the predictive value of cHER2 BC can be provided, by incorporating CSCs-driven intra-tumor heterogeneity into clinical decisions **(5).**

Consequently, the study aimed at investigating the proposal of involvement of the transition of CSCs biology and the molecular subtype in the development of trastuzumab resistance in HER2 +ve BC, recommending the use of trastuzumab combinations/modifications that are able to suppress CSCs and overcome trastuzumab resistance.

MATERIALS and METHODS

This retrospective study (2012-2015) was conducted in the departments of **Medical Oncology, Pathology, Biochemistry, Histology and Cell Biology, Faculty of Medicine, Cairo, Egypt** in collaboration with **Faculty of Pharmacy, Near East University North Cyprus**. The study was approved by the institutional review board.

The study was conducted on 40 BC patients age range 27-63 years (average 45 years), 20 served as +ve control group and 20 as metastatic (predicted resistance) group. All patients had BC confirmed with a microscopic examination with proven immunohistochemical (IHC) analysis of HER2 disease, in postoperative specimens or samples obtained by biopsy. All BC patients gave informed consent according to the institutional review board approved protocol.

I. Clinicopathological Analysis:

The study group comprised inclusion criteria for trastuzumab therapy and medical history without serious comorbid conditions such as unstable ischemic heart disease, valvular heart disease, chronic hypertension with cardiovascular problems or uncontrolled diabetes. Subsequent inclusion criteria were the peripheral blood count, renal and liver function within normal values. The data, including the age at onset, menopausal status, weight, height, the history of neoplasms in the first degree family and diabetes mellitus (DM) were recorded. Date of diagnosis, breast imaging, surgical procedures, tumor stage according to TNM classification, grade and proliferation index were collected. HER2 status, ER status, PR status, baseline ejection fraction (EF), chemotherapy regimen and number of herceptin cycles were gathered from hospital records and pathology reports.

All tumor paraffin embedded specimens were assigned a study identification number that is distinct from the patient's medical record number.

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The histological type and grade of invasive disease were coded according to the TNM classification system. Tumor specimens were that have a 3+ stain intensity on **IHC analysis** were considered HER2 +ve.

Disease status was evaluated every 6-12 weeks using the same imaging techniques and the treatment schedule until end of follow-up or relapse (development of resistance). The 40 HER2 +ve BC patients received trastuzumab treatment and the predicted resistance to treatment was further investigated for by:

II. Histological Analysis:

Tripple immunostaining was performed for control and predicted resistance cases. Negative (-ve) control sections were obtained from safety margin.

A. Molecular Subtypes Markers:

HER2 **(6)** [0.2 ml diluted primary antibody (Ab) **(c-erbB-2 oncoprotein)** (A048529) (Dako)] and cytokeratin (CK) 5/6 immnostaining **(7)** [0.1 ml prediluted primary Ab **(CK5/6)** (M7237) (Dako)] in Histology and Cell Biology Department. HER2 reaction is membranous/ cytoplasmic. Cellular localization is cytoplasmic for cytokeratin 5/6. Human tonsil was used as +ve control section for cytokeratin 5/6. On the other hand, two of the BC sections were used as negative controls by omitting the step of applying the primary antibodies.

B. Cancer Stem Cell Marker:

A mesencymal subset of CSCs was defined as cells with a CD44+ve immunostaining **(8)** [0.1 ml diluted primary Ab **(CD44)** (IW-PA1021) (IHW, Ellicott City, USA)] in Histology and Cell Biology Department. Cellular localization is membranous for CD44. Human tonsil was used as +ve control section for CD44 Ab. On the other hand, one of the BC sections was used as -ve control by omitting the step of applying the primary Ab.

C. Morphometric Study:

Using **Leica Qwin 500 LTD** (Cambridge UK) computer assisted image analysis, assessment of area% of CD44 +ve cells, that of HER2 and that of CK5/6 +ve immunoexpression in the BC immunostained sections were performed. The measurements were done in 10 high power fields in control and sections of predicted resistant cases using binary mode.

III. Biochemical Analysis:

Evaluation of formalin-fixed paraffin-embedded (FFPE) BC specimens by **real time quantitative polymerase chain reaction** (qPCR) was performed **(9)** in Biochemistry Department. Paraffin-embedded tumor tissues collected from 40 patients, samples where tumor cells comprised 70% of the sample were selected. Reverse Transcription is carried out with the SuperScript First-Strand Synthesis System for reverse transcriptase (RT)-PCR. The following procedure is based on Invitrogen's protocol. The following ribonucleic acid (RNA)/primer (5'ATGAGCTACCTGGAGGATGT 3' 5'CCAGCCCGAAGTCTGTAATTT 3') (mixture was prepared in each tube: 5µg total RNA and 3 µl random hexamers). The samples were incubated at 65°C for 5 min and then on ice for at least 1 min. Reaction master mixture was prepared for each reaction then added to the RNA/primer mixture, mix briefly, and then placed at room temperature for 2 minutes. 1 µl (50 units) of SuperScript II RT was added to each tube, mixed and incubated at 25°C for 10 min. The tubes were incubated at 42°C for 50 min, heat inactivated at 70°C for 15 min, and then chilled on ice. 1 µl RNAase H was added and incubated at 37°C for 20 min. The 1st strand cyclic deoxyribonucleic acid (cDNA) was stored at -20°C until use for real-time PCR.

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The primer concentrations were normalized, gene-specific and reverse primer pair were mixed. Each primer (forward or reverse) concentration in the mixture is 5 pmol/µl. The PCR program was set up on ABI Prism standard deviation score (SDS) 7000. A copy of the setup file was saved and all PCR cycles were deleted (used for later dissociation curve analysis). 50°C 2 min, 1 cycle, 95°C 10 min, 1 cycle, 95 °C 15 seconds -> 60 °C 30 seconds -> 72 °C 30 seconds, 40 cycles and 72°C 10 min, 1 cycle. A real-time PCR reaction mixture can be either 50 µl or 25 µl. After PCR is finished, the tubes were removed from the machine. The PCR specificity was examined by 3% agarose gel using 5 µl from each reaction. Dissociation curve analysis was performed with the saved copy of the setup file. The real-time PCR result was analyzed with the SDS 7000 software.

IV. Statistical Analysis:

A P value of <0.05 was deemed statistically significant by using paired student's t- test. All statistical analyses were conducted using **Statistical Package for the Social Sciences** (SPSS) 16 software in Histology and Cell Biology Department **(10)**.

RESULTS

I. Clinicopathological Results:

In the present study, the analysis included 20 control BC cases and 20 metastatic (predicted resistant) BC cases treated with trastuzumab. The mean age in the control group at diagnosis was (45.35±10.50) and in the metastatic group it was (43.95±10.92), denoting a nonsignificant difference between both groups. Similarly, the mean weight and the mean height in control group at diagnosis was (76.20±9.80) and (159.1±19.18). In the metastatic group the mean was (82.00±23.29) and (157.50±13.98), denoting a nonsignificant difference between both groups. On the other hand, the mean proliferative index (PI) in control group at diagnosis was (20.55±2.54) and in the metastatic group it was higher (37.00±4.36), denoting a significant difference (P<0.05) between both groups. As regards the mean of ER positivity (+vity) in control group at diagnosis it was (0.60±0.17) and in the metastatic group it was much higher (3.90±0.60), denoting a significant difference (P<0.05) between both groups. In the +ve control group, the mean number of cycles was (8.15±1.32) and in the metastatic group it was (7.10±0.96) without significance **(Table 1)**.

Table 1: Mean ± standard deviation (SD) of clinicopathological results

Group	Age	Weight	Height	PI	ER+vity	Cycles
+ve Control	45.35± 10.50	76.20± 9.80	159.1± 19.18	20.55± 2.54	0.60± 0.17	8.15± 1.32
Metastatic	43.95± 10.92	82.00± 23.29	157.50± 13.98	37.00± 4.36*	3.90± 0.60*	7.10± 0.96

* significant P<0.05

II. Histological results

A. Molecular subtype markers:

HER2 immunostained sections demonstrated -ve immunostaining in the -ve control **(Fig 1 b)**, while in the +ve control group, obvious +ve IE was seen among the malignant cells **(Fig 1 b).** On the other hand, in the

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metastatic group, less obvious IE appeared in the specimens of cases with low PI, low ER and PR +vity **(Fig 1c)**. In addition, minimal IE was detected in the specimens of patients with high PI, high ER and PR +vity **(Fig 1d).**

CK immunostained sections demonstrated few +ve myoepithelial cells around ducts in –ve control **(Fig 2a)**, while +ve control cases revealed -ve IE among the malignant cells **(Fig 2b).** In the metastatic group, some of the cases with low PI, low ER and PR +vity revealed +ve CK IE in few malignant cells **(Figs 3a and 3b).** On the other hand, obvious IE was detected in multiple malignant cells lining ducts or forming solid masses in the specimens of patients with high PI, high ER and PR +vity **(Figs 3c and 3b).**

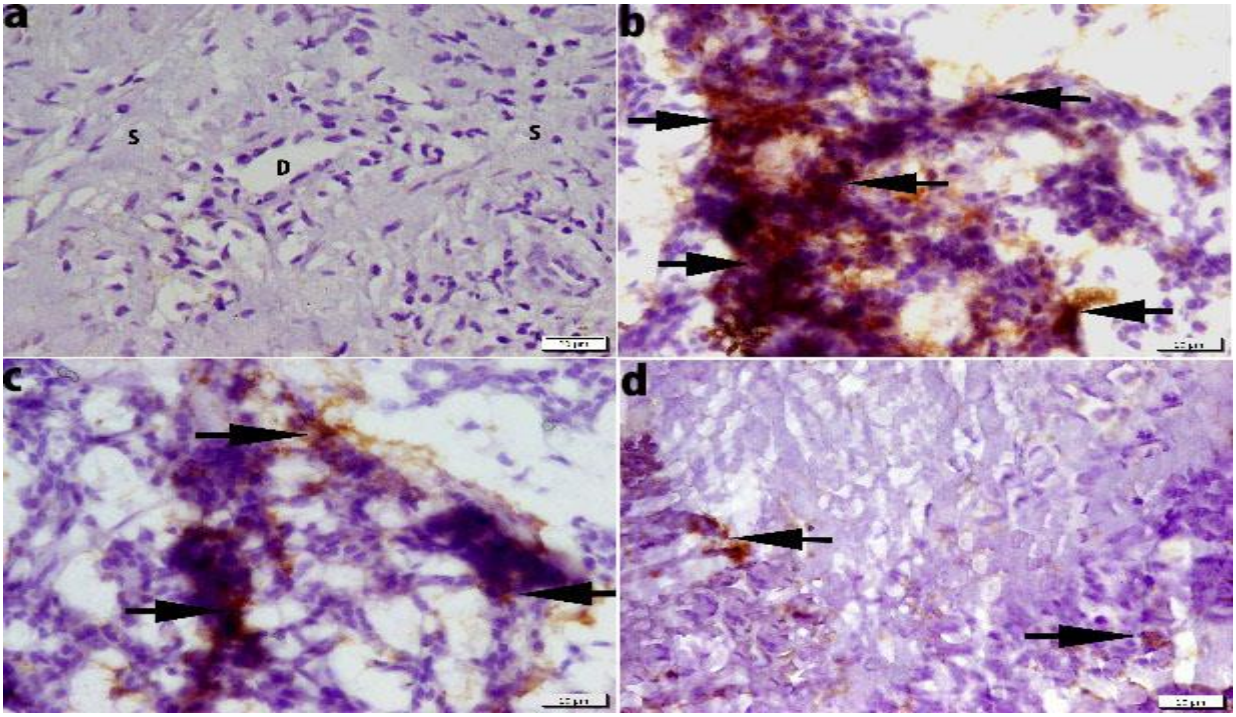

Fig 1: Photomicrographs of sections in (HER2 immunostaining, x400). **a:** breast tissue of -ve control showing -ve IE in the lining of a duct (D) and the surrounding CT. **b:** BC biopsy of the +ve control group showing obvious HER2+ve IE (arrows). **c:** BC biopsy of the metastatic group showing less obvious HER2+ve IE (arrows). **d:** BC biopsy of the metastatic group showing minimal HER2+ve IE (arrows).

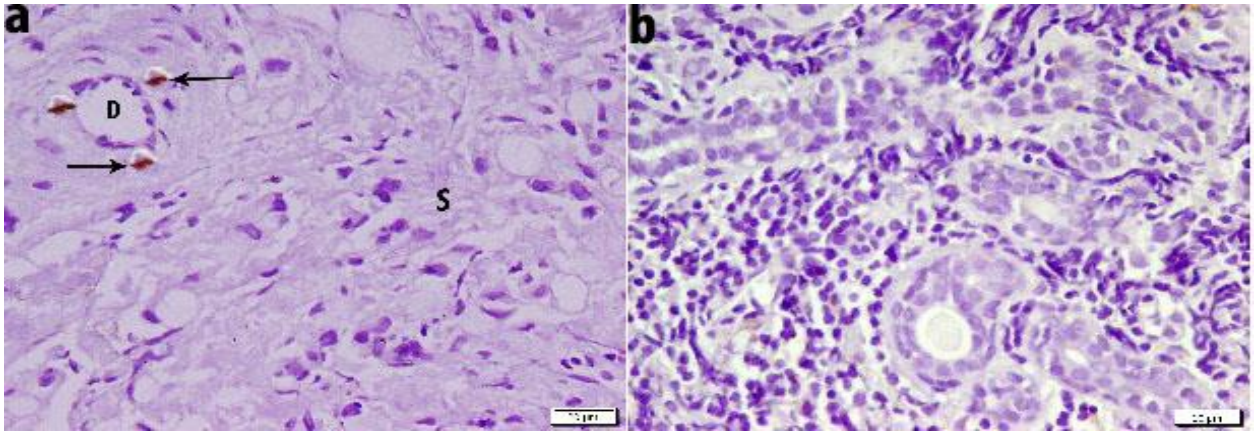

Fig 2: Photomicrographs of sections in (CK immunostaining, x400). a: breast tissue of -ve control showing few +ve myoepithelial cells (arrows) around a duct (D) and -ve IE in the surrounding CT (S). **b:** BC biopsy of the +ve control group showing -ve IE among the malignant cells.

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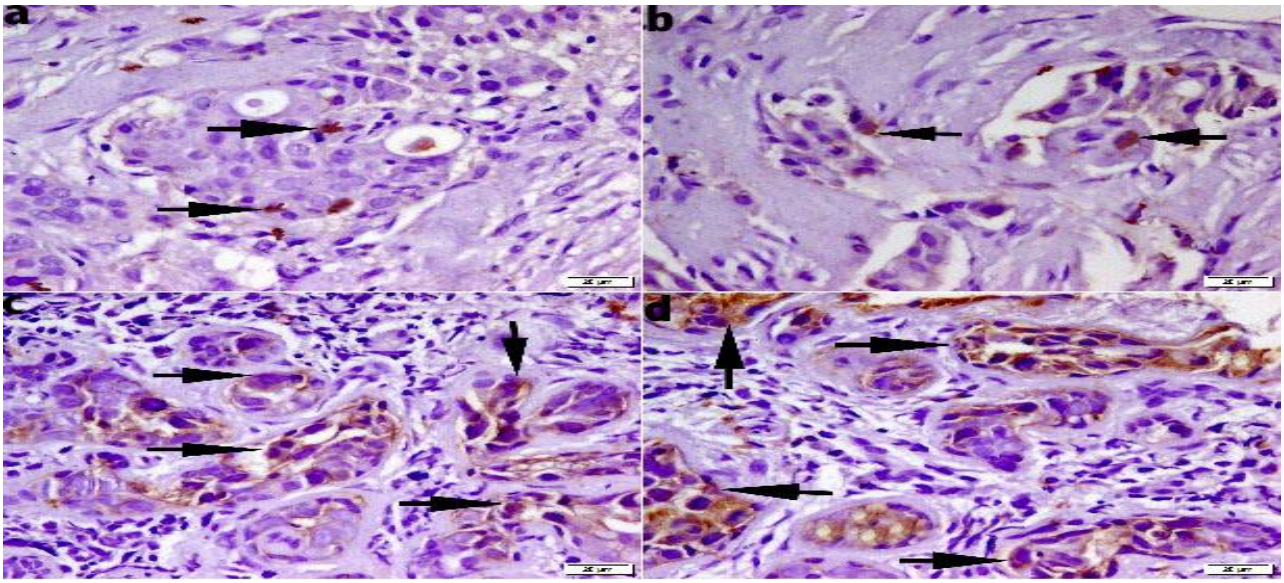

Fig 3: Photomicrographs of sections in (CK immunostaining, x400). a: BC biopsy of the metastatic group with low PI and low ER +vity showing +ve CK IE in few malignant cells (arrows). **b:** BC biopsy of the metastatic group with low PI, low ER and PR +vity showing +ve CK IE in few malignant cells (arrows). **c:** BC biopsy of the metastatic group with high PI, high ER and PR +vity showing obvious IE in multiple malignant cells lining ducts or forming solid masses (arrows). **d:** BC biopsy of the metastatic group with high PI, high ER and PR +vity showing obvious IE in multiple malignant cells lining ducts or forming solid masses (arrows).

B. Cancer Stem Cell Marker:

CD44 immunostained sections demonstrated -ve immunostaining in -ve control sections, while in +ve control group few +ve spindle cells were evident among the malignant cells **(Fig 4a).** While in the metastatic group, some of the cases with low PI, low ER and PR +vity revealed some +ve spindle cells among the malignant cells lining ducts and in the surrounding connective tissue (CT) **(Fig 4b)**. On the other hand, specimens of patients with high PI, high ER and PR +vity recruited multiple +ve spindle cells among the malignant cells lining a duct and in the surrounding CT **(Fig 4c).** In addition, multiple +ve spindle cells were found inside and near blood vessels **(Fig 4d)**.

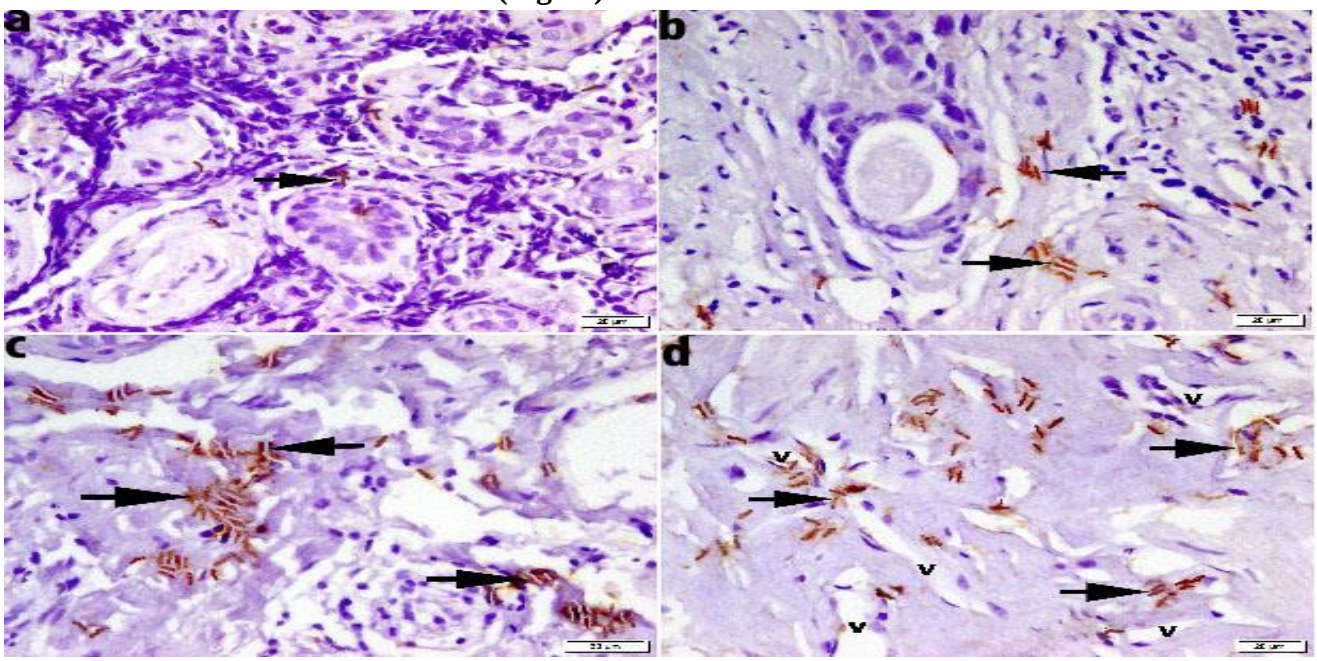

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Fig 4: Photomicrographs of sections in (CD44 immunostaining, x400). a: BC biopsy of the +ve control group showing few +ve spindle cells among the malignant cells (arrows). **b:** BC biopsy of the metastatic group with low PI, low ER and PR +vity showing some +ve spindle cells among the malignant cells lining ducts and in the surrounding CT (arrows). **c:** BC biopsy of the metastatic group with high PI, high ER and PR +vity showing multiple +ve spindle cells among the malignant cells lining a duct and in the surrounding CT (arrows). **d:** BC biopsy of the metastatic group with high PI, high ER and PR +vity showing multiple +ve spindle cells (arrows) inside and near blood vessels (v).

C. Morphometric results

The mean area% of HER2 +ve IE was (**16.01±0.76**) and (**9.11±1.36**) in the +ve control and in the metastatic groups respectively, indicating a significant decrease in the mean area% (P<0.05) **(Table 2)**. On the other hand, the mean area% of CK IE was (**0.12±0.02**) in the -ve control group. In the metastatic group +ve IE in cases with lower PI and low intensity of ER and PR IE was (**2.22±0.42**) and that in cases with high PI and high ER and PR +vity was (**5.08±0.45**), indicating a significant increase in the mean area% (P<0.05) in the metastatic group compared to -ve control group and also in cases with high PI and high ER and PR +vity compared to those with lower values (P<0.05). While, the mean area% of CD44 IE was **(0.84±0.21)** in the +ve control group. In the metastatic group, +ve IE in cases with lower PI and low intensity of ER and PR IE was (**4.34±0.48**) and that in cases with high PI and high ER and PR +vity was (**8.65±0.89)**, indicating a significant increase in the metastatic group compared to +ve control group and also in cases with high PI and high ER and PR +vity compared to those with lower values (P<0.05) **(Table 3)**.

III.Biochemical Results:

The mean values of PCR were **(8.82±1.17)** and **(2.77±1.11)** in the +ve control and the metastatic groups respectively, indicating a significant decrease in the metastatic group (P<0.05) **(Table 2)**.

Table 2: Mean ± SD of PCR values and HER2 +ve IE

Group	PCR values	HER2 +ve IE
+veControl	8.82±1.17	16.01±0.76
Metastatic	2.77±1.11*	9.11±1.36*

* significant (P<0.05) decrease compared to the control group

Table 3: Mean ± SD of CK +ve and CD44 +ve IE

Group	CK +ve IE	CD44+ve IE
-ve Control	0.12±0.02	-
+ve Control	-	0.84±0.21
Metastatic (low PI, ER and PR)	2.22±0.42#	4.34±0.48$
Metastatic (high PI, ER and PR)	5.08±0.45##	8.65±0.89$$

significant (P<0.05) increase compared to -ve control

significant (P<0.05) increase compared to -ve control and metastatic (low PI, ER and PR)

$ significant (P<0.05) increase compared to +ve control

$$ significant (P<0.05) increase compared to +ve control and metastatic (low PI, ER and PR)

Discussion:

The present study aimed at investigating the transition in the molecular subtype and CSCs biology of BC and relation to the development of trastuzumab resistance in HER2 +ve BC. By investigating 20 +ve control and 20 metastatic BC cases depending on clinicopathological data that proved significant differences in the metastatic group. In addition, immunohistochemical, morphometric and biochemical analysis of the specimens revealed significant changes.

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As regards the clinicopathological results, nonsignificant difference was found in the mean age, the mean weight, the mean height and the mean number of cycles between both groups. It can be commented that none of the previous data were proved to be directly related to the possibility of development of trastuzumab resistance in the metastatic group of BC versus the control group. **Santa-Maria et al (11)** reported that a better understanding of HER2 biology has led to the development of powerful targeted therapies. Four drugs were approved by the US Food and Drug Administration for treatment in the metastatic setting (trastuzumab, pertuzumab, lapatinib, and trastuzumab emtansine). However, while the prognosis has improved, metastatic disease is still not curable; newer, better drugs are needed. Novel therapeutics, include small-molecule inhibitors, nanoparticles, immunotherapy, and agents targeting resistance pathways. On the other hand, **Harbeck et al (12)** recorded that trastuzumab-based therapy remains the treatment of choice for patients with HER2-+ve metastatic BC who had progressed on trastuzumab.

On the other hand, in the present study, a significant increase in the mean proliferative index and the mean of ER +vity was detected in the metastatic group compared to the +ve control group. PR was +ve in 15 cases in the metastatic group and -ve in all cases of the control group. **Chen et al (13)** recorded that by the establishment of resistance to trastuzumab therapy, BC shift molecular phenotype to become more ER +ve, PR +ve and less HER2 +ve. **Balmativola et al (14)** documented that the record of the clinico-pathological findings (histological type and grade; estrogen, progesterone receptors, and HER2 status, Ki67, mitotic count) and data regarding the pathological response in corresponding surgical resection specimens was performed. The distribution of the mitotic numbers and the % of Ki67 were high in nonresponders to chemotherapy. **You et al, (15)** confirmed that the chemotherapeutic effect on postoperative recurrence and metastasis in tumor-bearing mice is evaluated by Ki67 immunohistochemistry.

In the +ve control group, **HER2 immunostained sections** demonstrated obvious +ve IE. While in the metastatic group, less obvious IE appeared in the specimens of cases with lower PI and low intensity of ER and PR IE. On the other hand, minimal IE was detected in the specimens of patients with high PI, high ER +vity and PR +vity. This was confirmed by a significant decrease in the mean area% of HER2 +ve IE in the metastatic group compared to the control group. The previous results may indicate a change in the molecular subtype of the HER2 +ve BC. Going with, **De Mattos-Arruda et al (16)** documented that a comprehensive molecular understanding of the pathways associated with resistance to trastuzumab and chemotherapy might greatly aid the development of more effective targeted therapies for patients with HER2-amplified BC. **Burnett et al (4)** added that the transformed resistant cells in HER2 +ve BC may exhibit loss of dependence on HER2 family signalling, which may increase the frequency of CSCs and metastasis potential.

In the +ve control group, **CK immunostained sections** demonstrated -ve IE among the malignant cells. While in the metastatic group, some of the cases with lower PI and low ER and PR +vity revealed +ve CK IE in few malignant cells. On the other hand, obvious IE was detected in multiple malignant cells lining ducts or forming solid masses in the specimens of patients with high PI, high ER and PR +vity. This was confirmed by a significant increase in the mean area% of CK IE in the metastatic group cases with higher PI and high ER and PR +vity compared to cases with low PI and low ER and PR +vity. In accordance, it was recorded that a basal-HER2+ phenotype was established solely on expression of the basal marker CK5/6 **(5)**. It was added that HER2+ tumors enriched with molecular and morpho-immunohistochemical features classically

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ascribed to basal-like tumors are highly aggressive and refractory to trastuzumab. Co-expression of HER2 protein and basal cytokeratin markers CK5/6 refers to basal- HER2+ phenotype **(17)**. Basal-like BC as an aggressive phenotype of breast malignancies was confirmed to be associated with poor prognosis **(18)**.

In the +ve control group, **CD44 immunostained sections** demonstrated few +ve cells among the malignant cells**.** While in the metastatic group, some of the cases with lower PI and low ER and PR +vity revealed some +ve cells among the malignant cells lining ducts and in the surrounding CT. On the other hand, specimens of patients with high PI, high ER and PR +vity recruited multiple +ve spindle cells among the malignant cells lining a duct, in the surrounding CT**,** inside and near blood vessels. This was confirmed by a significant increase in the mean area% of CD44 IE in the metastatic cases with low PI, low ER and PR +vity compared to the control group, also in cases with high PI, high ER and PR +vity compared to low PI, ER and PR +vity.

CD44 was proved to influence the P-glycoprotein-mediated multidrug resistance. This is mediated by prevention of accumulation of anticancer drugs within cells by virtue of its active drug efflux capacity **(19)**. It was reviewed that CSCs have been associated with metastasis and therapeutic resistance and can be generated via epithelial mesenchymal transition (EMT). The EMT is a mechanism to generate CSCs endowed with an invasive and metastatic phenotype. This process is mediated by the activity of growth and transcription factors, resulting in loss of the epithelial cells' typical intercellular junction structure, acquisition of mesenchymal morphology and invasion ability **(20)**. It was commented on CSCs, been invoked in resistance, recurrence and metastasis of cancer, consequently, curative cancer treatments may be concentrating on CSC selective approaches **(21)**.

In addition, the mean values of PCR indicated a significant decrease in the HER2 gene expression in the metastatic group compared to the +ve control group. It was stated that real-time PCR analysis indicated the measurement of miR-184 expression level that promotes tumor progression in lung cancer **(22)**. Similarly, **(23)** reported that RT-qPCR is a precise and cost-effective diagnostic approach for HER2 testing in cancer. The PCR assay is simple, accurate and robust and can easily be implemented and standardized in clinical laboratories.

It can be concluded that the development of resistance to trastuzumab in the metastatic group of HER2+ve BC is related to the development of the basal like subtype and the associated overexpression of CD44 +ve CSCs. This nictitates performing IHC assessment of the molecular subtype and the type of CSCs to allow the choice of the most appropriate treatment regimen giving the best expected prognosis.

Potential conflict of interest

The authors have no conflicting financial interest.

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