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Correlation between Serum and Tissue Markers in Breast Cancer Iraqi Patients

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Abstract:

Breast cancer is the most prevalent malignancy among women worldwide, in Iraq it ranks the first among the population and the leading cause of cancer related female mortality. This study is designed to investigate the correlations between serum and tissue markers in order to clarify their role in progression or regression breast cancer. Tumor Markers are groups of substances, mainly proteins, produced from cancer cell or from other cells in the body in response to tumor. The study was carried out from April 2018 to April 2019 with total number of 60 breast cancer women. The blood samples were collected from breast cancer women in postoperative and pretherapeutic who attended teaching oncology hospital of the medical city in Baghdad and the serum markers evaluated by ELISA technique are Carbohydrate Antigen 15-3 (Ca 15-3), Carbohydrate Antigen 27.29 (Ca 27.29), Anti-Mullerian Hormone (AMH), Tumor Necrosis Factor-Alpha (TNF- α), Interleukin-6 (IL-6), Interleukin-10 (IL-10) and Human Epididymis Protein-4 (HE4). Tissue samples were collected for the same breast cancer women who attended medical city, Baghdad with total number 30. The tissue markers evaluated by Immunohistochemical technique are Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (Her 2/neu) and Cyclin E. The results showed a positive significant correlation ($p = 0.017$) between Ca 27.29 and Her-2/neu, ($p = 0.038$) between IL-6 and cyclin E phenotype, ($p = 0.051$) between TNF- α and Cyclin E intensity, ($p = 0.005$) between HE4 and Her-2/neu, and negative significant correlation ($p = 0.058$) between IL-10 and ER score and ($p = 0.045$) between HE4 with Cyclin E intensity. We conclude from these correlations that positive correlations increasing disease progression, like correlation between Ca 27.29 and Her-2/neu, cyclin E with IL-6 and cyclin E with TNF- α . And the negative correlations may contribute to delay disease, like correlation between IL-10 and ER. From the correlations results in this study, it is clear that the Ca 27.29, Her-2 / neu, cyclin E markers play an important role in disease progression.

Keywords: Anti-Mullerian hormone (AMH), Breast cancer, Ca 15-3, Estrogen Receptor(ER), Human Epididymis Protein-4(HE4), Tumor Necrosis Factor-Alpha (TNF- α).

Introduction:

Breast cancer is one of the most common cancers in women. It is the most diagnosed cancer that causes death in women with a higher incidence in developed countries and higher death rates in developing countries^{1,2}. In Iraq, breast cancer ranks the first among the women and the leading cause of cancer related female mortality. Numerous studies from Iraq indicated that up to the present time a considerable rate of female patients still present with breast cancer at younger age groups with more advanced stages and aggressive tumor compared to their western counterparts. Ultimately leading to

undesirable prognosis³. In Iraq, about 34.27 % of new cases register for breast cancer in female in Iraqi women until 2016⁴.

Carbohydrate antigen Ca15 – 3, is a glycoprotein antigen. It is found on surface of cancer cell and sheds into blood stream and can be measured in saliva that is considered as blood stream in oral cavity that can be used for the detection of cancer begin and follow – up⁵. CA 15-3 is useful for determining prognosis of breast cancer and also for monitoring the efficacy of the treatment since the elevated level of this tumor

marker in serum tend to increase the severity of breast cancer⁶.

Tumor marker Ca 27.29 is a carbohydrate – containing protein antigen that reported as a tumor marker for breast cancer and it is called “ Breast Carcinoma-associated antigen”⁷. The Ca 27.29 considered is the tumor marker which breast cancer cell shed copies from Ca 27.29 protein to blood stream⁸.

Anti – Mullerian hormone (AMH, also called a Mullerian inhibiting substances (MIS) or Mullerian inhibiting factor) was described at first in 1947 by Alfred Jost⁹. It is produced by Sertoli cell in testis and it has a role during embryogenesis by inhibiting the development of Mullerian duct in male embryo so, it plays a role in male sex differentiation¹⁰. The AMH activity is exerted by two receptors: Type I receptor and Type II receptor present on the AMH target organs such as gonads and Mullerian ducts¹¹. The AMH type II receptor expressed in normal and tumor tissue in breast and limited laboratory finding suggest a protective role of AMH in breast carcinogenesis¹². In other study showed the lowered concentrations of AMH in breast cancer patients and chemotherapy have a high effect on AMH level¹³.

Tumor Necrosis factor alpha (TNF – α) is an inflammatory cytokine synthesized and secreted by many cell types especially macrophage, natural killer cell (NK) and T – cell. This cytokine has a central role in promoting inflammation, endothelial activation and has a significant role in cancer pathogenesis¹⁴. The TNF – α is expressed by normal breast epithelial cells, but high level expressed in tumor cells from most breast cancer patients and the high level of TNF – α correlated with recurrence and advanced disease¹⁵. The TNF – α is a necrotic factor in tumor microenvironment that stimulates tumor growth and migration and the elevated level related with metastasis¹⁶. On the other hand, reports of the anti-proliferative and apoptotic effect of TNF- α on breast cancer have only been executed on the MCF-7 cell line¹⁷. Some studies have shown that TNF- α may have a double-edged role in angiogenesis, depending on the used doses. High doses of TNF- α inhibit angiogenesis in mice while low doses promote vascularization of the area. The anti-angiogenic effect of TNF- α is related with the down-regulation of adhesion molecules, while pro-angiogenic responses have been associated with the increased expression of VEGF, VEGFR, IL-8, and FGF. Therefore, low levels of TNF- α increase tumor growth, induce angiogenesis of several tumors in mice, and stimulate tumor-associated myeloid cells and the co-expression of endothelial and myeloid markers

with pro-angiogenic/pro-vascular properties¹⁸. Interleukin – 6 (IL – 6) is a pro-inflammatory cytokine, it is produced by macrophage and monocyte. IL – 6 is expressed by multiple tumor tissue like breast, prostate, colorectal and ovarian cancer¹⁹. An elevated serum level of IL – 6 is shown to be correlated with disease staging and undesirable outcomes in women with metastatic breast cancer and it has a role in tumor advancements²⁰. The IL – 6 has a central role in tumor behavior like tumor growth, cell proliferation, migration and invasion, angiogenesis and metastasis¹⁹.

Anti – inflammatory cytokine, (IL – 10) gene are located on chromosome 1 at q31-32. It is produced by immune cell like T – lymphocyte, macrophage and natural killer cell (NK)²¹. It has an important role in breast carcinogenesis. IL-10 might promote tumor development; by suppress anti-tumor immune responses²².

Human Epididymis protein – 4 (HE 4) discovered by Kirchoff *et al*, in 1991 was first identified in males in distal epithelium of Epididymis²³. It has 124-amino acid long polypeptide²⁴. The HE 4 gene is localized on human chromosome 20q12 – 13²³. The HE 4 is reported expressed in many normal and malignant tissues, breast epithelium, female genital tract, Epididymis, vas deference, distal renal tubules, respiratory epithelium, colon mucosa and salivary glands all show HE 4 immuno-reactivity²⁵. The HE 4 is expressed in ductal carcinoma of breast cancer tissue²⁶.

Estrogen and progesterone receptors are hormone receptors found on breast cells that pick up hormone signals resulting in cell growth²⁷. In breast cancer status the determination of these receptors is useful for therapeutic options and provide prognostic information²⁸. Human epidermal growth factor receptor (Her – 2) was first discovered in 1984 by Weinberg and associated²⁹. The gene that encoded to this receptor is situated on chromosome 17q³⁰. It is called as a neu in rat, the gene expression result production 185kDa transmembrane glycoprotein known as her – 2 proteins and have extracellular domain (ECD)³¹.

Cyclin E / *CCNE1* gene is localized on chromosome 19q³². Cyclin E is nuclear protein identified through it is capacity of division abnormality in cyclin – deficient yeast cells. Cyclin E has a high expression in many human cancers including breast, endometrium, lung, cervix, gastrointestinal tract and lymphoma. The high cyclin E expression was prognostic factor which correlated with worse patient outcome in women with breast cancer³³.

This research discusses a group of serum and tissue markers in a group of Iraqi women with breast cancer. The research clarifies the correlations between these markers because of their importance in the progression of the disease or its regression.

Materials and Methods:

Sixty patients (32-75 years old) who admitted the Oncology Teaching hospital, Medical City-Baghdad confirmed as breast cancer by physical examination, biopsy tacking, ultrasound and mammogram. Postoperatively patients followed – up for defining the histopathology classification of breast cancer stage and lymph node metastasis were recorded. The patients did not receive chemotherapy, radiotherapy or hormonal therapy yet.

Blood samples

The blood was collected from patients by 5 ml disposable syringe and then was put in gel clot activator tube and left room temperature to allow clotting after that entered to centrifugation 3000 rpm for 15 minutes then serum distributed in 4 eppendorf tubes in equal amount and stored in deep freeze until time to use.

Kits used

The all serum biomarkers (Ca 15-3, Ca 27.29, AMH, IL-6, IL-10, TNF- α and HE4) for breast cancer women were evaluated depending on sandwich ELISA principles using three antibodies, capture antibody, detection antibody and HRP-linked secondary antibody. The sample with unknown amount of antigens is immobilized on wells of the plate by bind with capture antibody. The antigen-antibody complex is linked to an enzyme-bound secondary antibody. In the final step a substrate is added that the enzyme can convert to some detectable signal like color³⁴. The Ca15-3 kit purchased from Thermofisher (USA), Ca27.29 kit from Mybiosource (USA). The AMH kit is from Kamiya biomedical (USA). The TNF- α , IL-6 and IL-10 kits are from Thermofisher (Austria). The HE4 kit is from Cusabio (China). Tissue markers were evaluated by Immunohistochemical technique. The ER, PR and Her-2/neu kits from Zytomed system (Germany) and cyclin E kit from Bio SB (USA). The method of action of the serum markers was according to was mentioned in the kit used for each marker.

Clinico-pathological Features

The clinic-pathological features of patients female are illustrated in Tables 1 and 2.

Table 1. Illustrated the patient ages and body mass index (BMI) means for patients studied.

Two sample t test showed differences between means of age and body mass index according to studied groups.

	Groups	N	Mean \pm S.D	P value
Age	Breast cancer women	60	52.20 \pm 9.940	0.532
	control	20	51.05 \pm 8.082	
Body mass index	Breast cancer women	60	31.22 \pm 5.852	0.188
	Control	20	29.30 \pm 4.703	

Table 2. Illustrated the patients children number, smoking, chronic disease, breast feeding, family History, type of tumor, stage and grade.

No	Studied Variables	Total Number (60)
1	Children Number	
	Yes (have children)	52
2	No(have no children)	8
	Smoking	
3	Yes(smoker)	3
	No(Non smoker)	57
4	Hypertension	
	Yes(have hypertension)	22
5	No(have not hypertension)	38
	Diabetes	
6	Yes(have diabetes)	8
	No(not have diabetes)	52
7	Breast Feeding	
	Yes(breast feed)	51
8	No(no breast feed)	9
	Oral Contraceptive pills	
9	Yes(taking contraceptive pills)	3
	No(not taking contraceptive pills)	57
10	Family History	
	Yes(have first-degree history)	3
11	No(have no first-degree history)	57
	Type of Tumor	
12	Ductal Carcinoma In Situ (DCIS)	4
	Invasive Ductal Carcinoma (IDC)	52
13	Invasive Lobular Carcinoma (ILC)	4
	Stage	
14	Stage I	6
	Stage II	28
15	Stage III	26
	Grade	
16	Grade I	2
	Grade II	43
17	Grade III	15

Tissue samples

The immunohistochemistry is a technique for identifying cellular or tissue antigens by mean antigen-antibody interactions. The IHC staining is complete by using enzyme-labeled (immunoperoxidase) antibodies to identify proteins.

The secondary antibody is reactive against the primary antibody conjugated or linked to an enzyme marker. Finally, the color of the reaction is determined by precipitating chromogen, usually Di Amino Benzidine (DAB) (brown color) with which the enzyme react³⁵. Tissue samples (Formalin Fixed Paraffin Embedded Tissue "FFPE") were collected from 30 patients who admitted to tissue laboratory, Oncology Teaching hospital, Medical City-Baghdad. The desired information about the patients and histopathological information of the tumors were collected from the patients' files. The tissue section slides of ER, PR, Her - 2 / neu and Cyclin E receptors were imaged by using light Microscope (leica) and camera (leica) by 10 x and 40 x objective lenses and magnification power at 100 X and 400 X.

Tissue Preparation for immunohistochemistry Reagents Preparation

- 1 - Reagents should be at room temperature when used.
- 2 - Deparaffinise and rehydrate paraffin-embedded tissue sections.
- 3 - Pre-treatment with HIER (Heat Induced Epitope Retrieval).
- 4 - The tissue sections have to be completely covered with the different reagents in order to avoid drying out.
- 5 - Preparation of the chromogenic substrate DAB working solution. Add 4 drops (200 µl) of DAB concentrate to one bottle of DAB substrate buffer and mix thoroughly.

Staining Procedure

The procedure of the ER, PR and Her2/neu by IHC assay in this study is carried out in accordance with the manufacturer instructions (Zytomed system, Germany).

- 1- Peroxide block (3% H₂O₂ solution).
- 2 -Washing with wash buffer.
- 3 -Primary antibody used.
- 4 -Washing with wash buffer.
- 5 -Biotinylated secondary antibody.
- 6 -Washing with wash buffer.
- 7 -Streptavidin-HRP-conjugate.
- 8 -Washing with wash buffer.
- 9 -DAB adding.
- 10 -Stopping the reaction with distilled water when the desired color intensity is attained.
- 11 -Counterstaining and blueing.
- 12 -Mounting: permanent with DAB.

The ER and PR section was read according Allred score (proportional percentage + intensity of staining) of tumor cells and according the intensity of staining cells for Her-2/neu and these reading

according to Iqbal BM and Buch A, 2016³⁶ (Tables 3 and 4).

Table 3. The Allred score (proportional + intensity) for ER and PR³⁶.

N	Allred score	Final result
1	0/8	Negative
2	1/8 – 2/8	Negative
3	3/8 – 4/8	+ Ve Weak
4	5/8 – 6 /8	+ Ve Moderate
5	7/8 – 8/8	+ Ve Strong

Table 4. The score of Her-2/neu depend on intensity of staining cells³⁶.

N	Staining Pattern	Score of Her-2
1	No staining the tumor cells < 10 %	0 -Ve
2	Faint / incomplete membrane staining in tumor cells > 10 %	+1 (-Ve)
3	Weak to moderate complete membrane staining in tumor cells > 10 %	+2 (+Ve)
4	Strong complete membrane staining in tumor cells > 10 %	+3 (+Ve)

Immunohistochemical reading for cyclin E protein is divided into two types according to Karakas C *et al*, 2016³⁷ (Tables 5 and 6).

Table 5. The first reading of cyclin E according to phenotype³⁷.

N	First reading according to Phenotype
1	Phenotype I = no nuclear and No cytoplasm
2	Phenotype II = + nuclear and No or weak cytoplasm
3	Phenotype III = + nuclear and + cytoplasm
4	Phenotype IV = no or weak nuclear and + cytoplasm

Table 6. The second reading of cyclin E according to intensity of tumor cells³⁷.

N	Second reading according to Intensity of tumor cells
1	No Stain
2	Weak
3	Intermediate
4	Strong

Statistical analysis

The statistical analysis was performed using the statistical package SPSS version 23. Results were considered at significant (P<0.05) difference levels. The correlation test was used to find out significance of correlation between related variables.

Results:

The mean of serum markers of patients samples are clarified in Table 7 and Fig. 1.

Table 7. The differences between serum markers according to studied groups.

Two sample t test showed differences between means of serum variables according to studied groups.				
Variables	Groups	N	Mean ± S.D	P value
Ca 15-3	Cases	60	35.450 ± 4.7136	0.001
(U / ml)	Control	20	8.180 ± 2.3210	
Ca 27.29	Cases	60	113.300 ± 17.3120	0.001
(U / ml)	Control	20	12.485 ± 4.8788	
AMH	Cases	60	0.6172 ± 0.17960	0.001
(ng / ml)	Control	20	2.0450 ± 0.44895	
IL-6	Cases	60	20.590 ± 5.0427	0.001
(pg / ml)	Control	20	4.405 ± 1.0086	
IL-10	Cases	60	8.793 ± 2.3440	0.001
(pg / ml)	Control	20	2.830 ± 0.7371	
TNF-α	Cases	60	25.963 ± 6.9211	0.001
(pg / ml)	Control	20	4.685 ± 1.5869	
HE4	Cases	60	91.247 ± 29.4110	0.001
(p mol / L)	Control	20	15.755 ± 5.5766	

❖ Ca 15-3 = Carbohydrate Antigen, Ca27.29 = Carbohydrate Antigen, AMH = Anti-Mullerian Hormone, (TNF-α) = Tumor Necrosis Factor Alpha, (IL-6) = Interleukin 6, (IL -10) = Interleukin 10, HE 4 = Human Epididymis Protein 4.

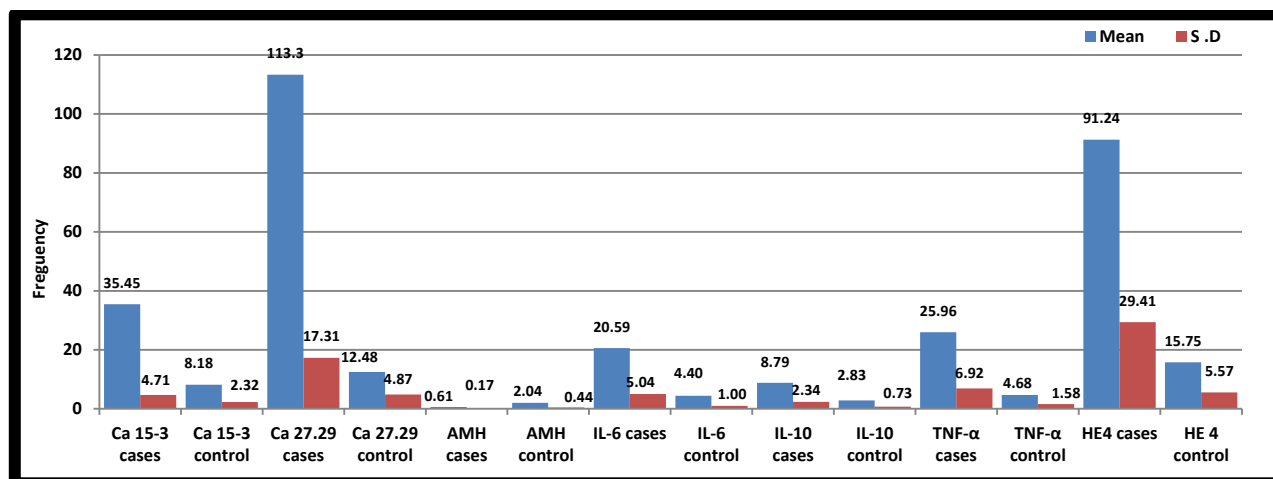


Figure 1. The graphs showing the means of serum markers for control and female patients.

This research showed that 70 % from cases were ER and PR positive as shown in Table 8. The Her-2/neu results showed 33% from cases were negative as shown in Table 9. While cyclin E phenotype showed 46% from cases were phenotype III and 33% from cases were strong staining cells as shown in Tables 10,11.

Table 8. The percentage of ER and PR tissue samples.

ER and PR Phenotype	Number	Percentage %
ER + / PR +	21	70
ER + / PR -	0	0
ER - / PR +	1	3.3
ER - / PR -	8	26.6
Total	30	100

Table 9. The percentage of Her-2/neu tissue samples.

Her - 2 / neu status	Number	Percentage %
Score 0 Negative	10	33.3
Score 1 Negative	6	20
Score 2 Positive	7	23.3
Score 3 Positive	7	23.3
Total	30	100

Table 10. The percentage of phenotype cyclin E tissue samples.

Cyclin E / phenotype	Number	Percentage %
Phenotype I	3	10
Phenotype II	11	36.6
Phenotype III	14	46.6
Phenotype IV	2	6.6
Total	30	100

Table 11. The percentage of intensity of cyclin E tissue samples.

Cyclin E intensity	Number	Percentage %
No stain	3	10
Weak	8	26.6
Intermediate	9	30
Strong	10	33.3
Total	30	100

The results revealed correlations between the means of serum markers and tissue markers in breast cancer patients. The results showed that was a positive significant correlation between Ca 27.29 with Her-2 P (0.017), HE 4 with Her-2 P (0.005), IL – 6 and cyclin E phenotype P (0.038), TNF – α with cyclin E intensity P (0.051) and revealed a negative significant correlation between IL – 10 and ER score P (0.058) and HE 4 with cyclin E intensity P (0.045) as in Table 12.

Table 12. The correlation between serum and tissue markers in studied breast cancer patients.

		ER score	PR score	Her-2/neu	Cyclin E Ph.	Cyclin E int.
Ca 15.3	CC	- 0.080	- 0.291	0.116	0.173	0.133
(U / ml)	Sig.	0.673	0.119	0.541	0.361	0.482
Ca 27.29	CC	0.082	- 0.011	0.432*	0.072	- 0.061
(U / ml)	Sig.	0.668	0.954	0.017	0.705	0.747
AMH	CC	0.117	0.181	- 0.234	0.006	- 0.021
(ng / ml)	Sig.	0.537	0.339	0.214	0.975	0.911
IL-6	CC	- 0.266	- 0.331	- 0.020	0.381*	0.060
(pg / ml)	Sig.	0.155	0.074	0.918	0.038	0.752
IL-10	CC	- 0.350	- 0.336	- 0.157	- 0.082	0.190
(pg / ml)	Sig.	0.058	0.069	0.406	0.667	0.314
TNF - α	CC	- 0.055	- 0.170	- 0.251	- 0.114	0.359
(pg / ml)	Sig.	0.774	0.368	0.180	0.547	0.051
HE 4	CC	0.032	0.153	0.495**	- 0.223	- 0.368*
(pmol / L)	Sig.	0.867	0.420	0.005	0.237	0.045

*CC = Correlation Coefficient

❖ Ca 15-3 = carbohydrate antigen, Ca27.29 carbohydrate antigen, AMH = Anti-Mullerian hormone, IL-6 = interleukin 6, IL -10 = interleukin 10, TNF- α = tumor necrosis factor alpha, HE 4 = human Epididymis protein 4, ER = estrogen receptor.

Tissue sections

In invasive breast carcinoma the negative status of estrogen receptor (ER) and progesterone receptor (PR) mean that no ER and PR expression (0%) staining cells and the nuclear staining < 1% of total cancer cells as illustrated in Fig. 2, while the levels of (ER) and (PR) expression were positively 70% (90-100%) from cancer cells reaction with immunostaining and strong intensity as showed in Figs 3 and 4.

Her-2/neu is a cell membrane receptor and its staining depends on the intensity of cancer cells staining. Figure 5a shows no cancer cells staining because these cancer cells did not express of her-2/neu receptor, while in Figs 5b and c respectively shows reaction between her-2/neu receptor with immunostaining.

The Fig. 6a shows the phenotype II of cyclin E expression that occur nuclear staining exceeded cytoplasmic staining and Fig. 6b the phenotype III of cyclin E that was nuclear and cytoplasmic staining are equal. Figure 6c shows the moderate staining of cancer cells because the moderate levels of cyclin E expression. Figure 6d reveals a strong staining due to the high levels of cyclin E expression in breast cancer cells.

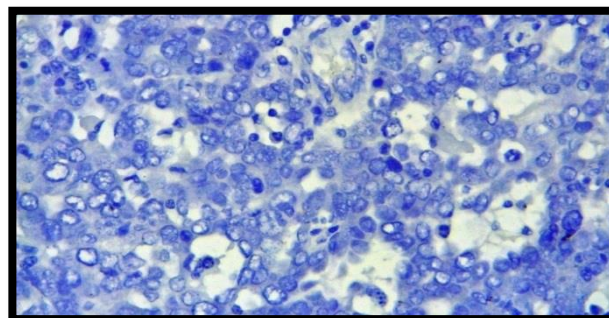


Figure 2. Poorly differentiated invasive breast ductal carcinoma showing negatively immunohistochemical of Estrogen receptor staining with background of counter stain.400 X.

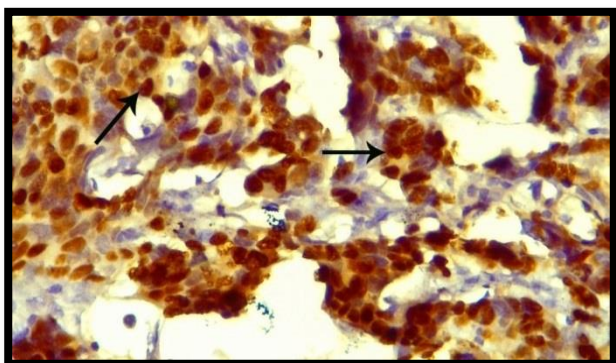


Figure 3. Moderately differentiated invasive breast ductal carcinoma. The arrow shows 95 % proportional positive immunohistochemical nuclear staining for Estrogen receptor antibody with strong intensity. 400 X.

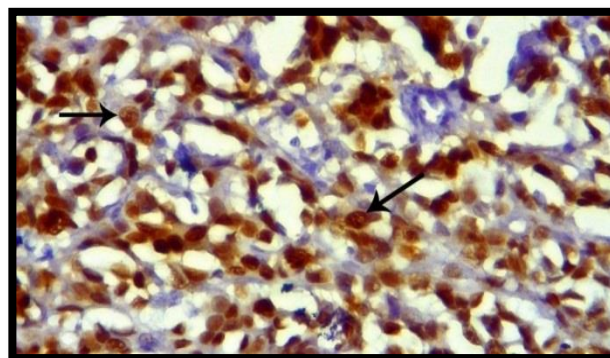


Figure 4. Poorly differentiated invasive breast ductal carcinoma. The arrow shows immunohistochemical nuclear staining for Progesterone receptor antibody about 90 % proportional with moderate intensity. 400 X.

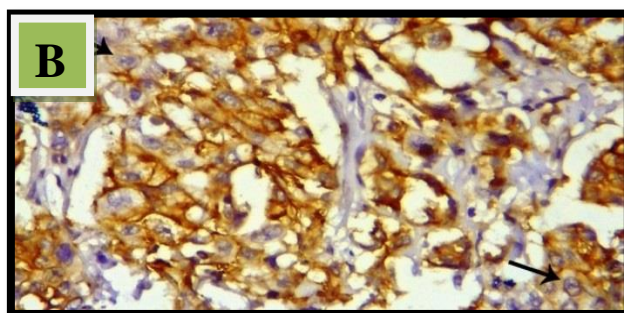
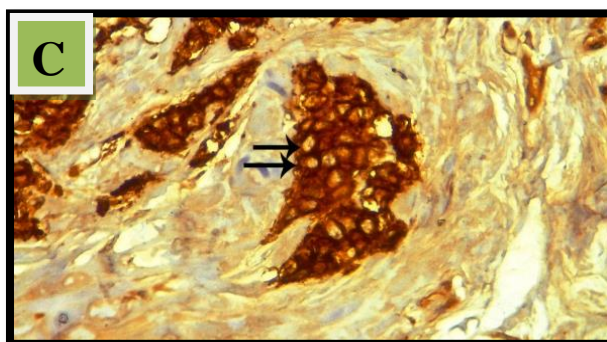
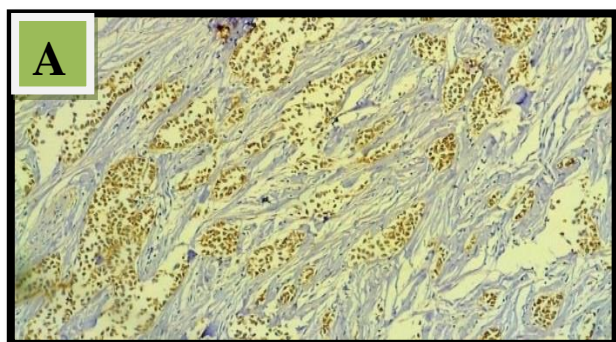


Figure 5. (A) Poorly differentiated invasive breast ductal carcinoma showing weak interrupted membranous staining for Her 2/neu antibody using IHC technique, more than 10 % of malignant cells score (-1) negative. 100 X. (B) Moderately differentiated invasive breast ductal carcinoma, the arrow shows moderately membranous staining for Her2/neu antibody by IHC technique for more than 10 % of malignant cells. Score (+2) equivocal (weak positive). 400 X. (C) Moderately differentiated invasive breast ductal carcinoma, the arrow shows a complete membranous staining for Her2/neu antibody using IHC technique for more than 10 % of malignant cells. Score (+3) (Strong positive). 400 X.

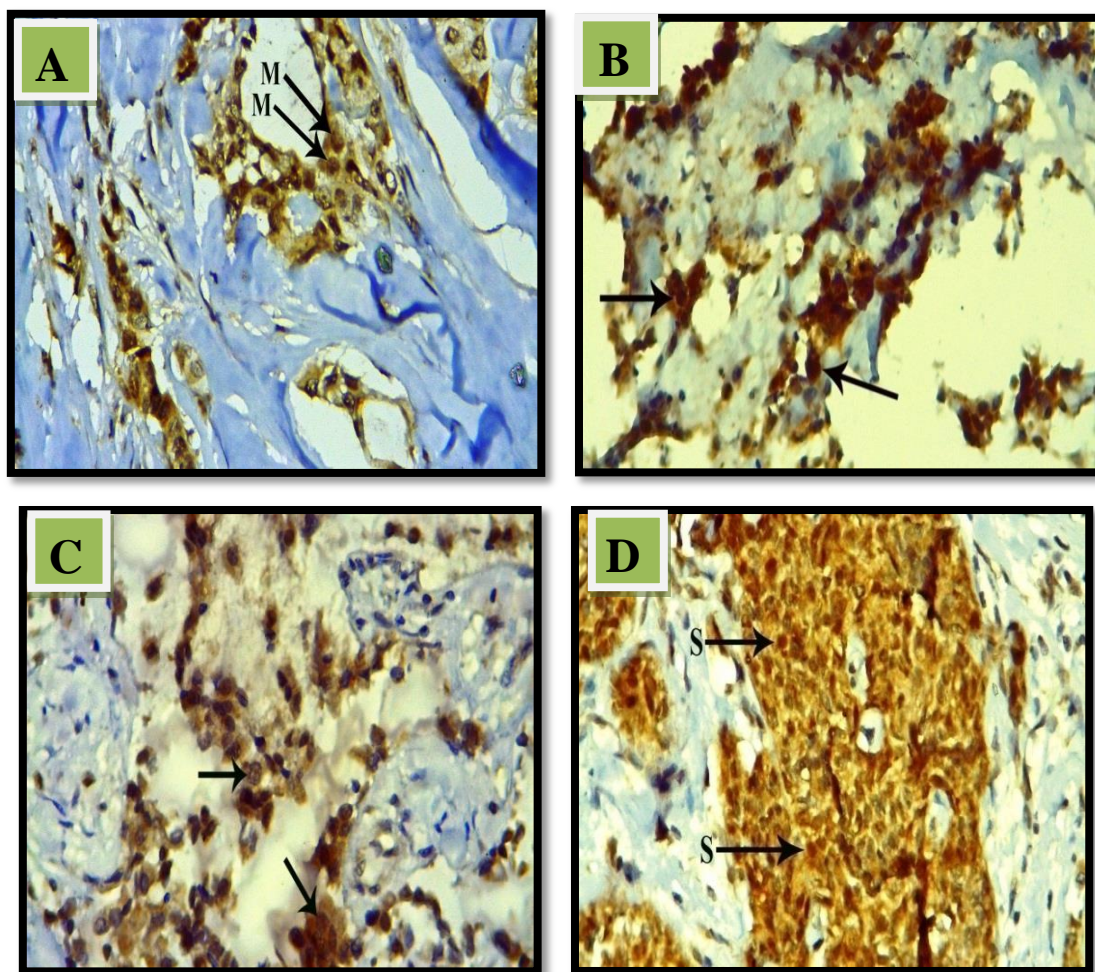


Figure 6. (A) Invasive breast ductal carcinoma, the arrow shows cyclin E phenotype II positive nuclear and weak cytoplasm. 400 X. (B) Invasive breast ductal carcinoma showing cyclin E antibody phenotype III positive nuclear and positive cytoplasm by using IHC technique. 400 X. (C) Moderately differentiated invasive breast ductal carcinoma showing cyclin E antibody by IHC technique. The arrow shows moderate (M) intensity. 400 X. (D) Moderately differentiated invasive breast ductal carcinoma showing cyclin E antibody using IHC technique. The arrow shows strong (S) intensity. 400 X.

Discussion:

This research explained that the correlations between serum and tissue markers have an important role in the progression or regression of breast cancer through the effect of each marker on the other marker. The negative correlation ($p < 0.05$) between ER and IL-10 in our research Corresponds with Zhuangwei *et al*, who reported a high serum IL-10 correlated with ER negative expression³⁸. Bhattacharjee *et al*, also revealed a significant correlation between IL-10 with ER and PR -ve tumors and IL-10 expression correlated with ER,PR -ve and Her-2/neu +ve significantly ($p = 0.01$) and this subtype of breast cancer (ER,PR -ve and Her-2 +ve) considered as the worst prognosis among the other subtypes³⁹. Sheikhpour *et al*, reported that IL-10 over-expressed in ER -ve and interpreted that expression of activator protein (AP - 1) is high in ER -ve in compared with ER +ve

due to increased AP-1 expression in related with IL-10²¹.

The AP-1 is transcription factor regulates gene transcription and it is implicated in regulating many physiological and pathological cellular process like proliferation, differentiation, growth, programmed cell death, cell migration and transformation. AP-1 mediated gene expression in response to inflammatory cytokine and it important for the pathogenesis of disease⁴⁰. Carruba *et al*, concluded that estrogen hormone (E2) is essential regulators of cytokine in cultured macrophage, the results reported that E2 induced decrease IL-10 secretion and that estrogen play a role in reduce IL-10 production⁴¹.

The positive correlation ($p < 0.05$) between Ca 27.29 and Her-2/neu markers in our study agrees with Yerushalmi *et al*, who explained a significant correlation between Ca 15-3 and Her-2/neu⁴². We

suggest the correlation between Ca 27.29 and Her-2/neu is similar the correlation between Ca 15-3 and Her-2/neu and that's because both of Ca 15-3 and Ca 27.29 are products for the same MUC1 gene⁴³. The experiments about breast cancer revealed that MUC1 RNA expression was the highest in Her-2/neu over-expression in tumor patients samples, the results reported that when treating cells with concentrated lapatinib (specific tyrosine kinase inhibitor of Her-2/neu) it causes a decrease in MUC1 expression and in another experiment on MDA-MB-361 cell line when treated cell with lapatinib showed that decrease Her-2/neu phosphorylation and a significant reduction in MUC1 levels⁴⁴. The Her-2 receptor is over-expressed in human breast cancer cells and the MUC1 associated with Her-2 at the surface of breast cancer cells, MUC1 formed from N-terminal (MUC1-N) and C-terminal (MUC1-C) fragments The MUC1-C associated with Her-2 receptor. The results revealed that MUC1-C cytoplasmic domain is sufficient to form complexes with Her-2 and this connection contributed to Her-2 activation and in carcinoma cells that suffered from epithelial-mesenchymal transition. The MUC1-C interact with Her-2 complex and promote activation of Her-2 by this way the breast cancer cells appear have sabotaged the physiological response to support their growth and survival⁴⁵.

The results of study revealed a positive correlation ($p < 0.05$) between HE 4 and Her-2 in breast cancer. This result agree with result of Akoz *et al*, on breast cancer between Her-2 expression and HE 4 staining intensity by immunohistochemical technique and demonstrated that in breast tumors, the increasing of Her-2/neu amplification was in parallel with HE 4 staining intensity, Her-2/neu amplification is known as poor prognostic factor for breast cancer and proved that HE 4 expression also unfavorable prognostic factor in breast cancer⁴⁶. The HE 4 protein enhance the proliferation, invasion and metastatic ovarian cancer cells and have the same biological effects on endometrial cancer, HE 4 can improve cell viability and found that HE 4 may play an essential role in EGFR (epidermal growth factor receptor) activation⁴⁷. The EGFR also known (Her-2), EGFR (Her-2) is over-expressed in breast carcinoma and this over-expression due to EGFR (Her-2) gene amplification that can observed in many cancer types like breast, lung, colorectal and esophageal cancers⁴⁸. So the increase of HE 4 in patients can induce to increase effectiveness EGFR (Her-2) that also increased in breast tumor.

The positive correlation ($p < 0.05$) between IL-6 and cyclin E agree with Wei *et al*, who demonstrated that IL-6 able to suppress cyclin – dependent kinase 2 (CDK 2) activity by accumulation P27^{kip1} protein⁴⁹. Pan, Y. and Claret, F. X. concluded that IL-6 interact with Jab1/Csn5 to regulate un-phosphorylated STAT3 and the decrease of Jab1/Csn5 expression caused decrease un-phosphorylated STAT3 and that STAT3 bind to Jab1/Csn5 to increased it promoter activity with increase Jab1/Csn5 transcription in breast cancer while, decrease STAT3 caused lowering Jab1/Csn5 promoter activity and Jab1/Csn5 protein expression. The IL-6 was important activators of STAT3 and contributed IL-6 to activation Jab1/Csn5 transcription and expression by STAT3⁵⁰. The Jab1/Csn5 gene is located on chromosome 8 and the expression of Jab1/Csn5 was found aberrantly expressed about 50 % in primary breast tumors and 90 % in metastatic lesions⁵¹. The Jab1/Csn5 over-expression is negatively correlated with P27 expression and that P27 can inhibit CDK and suppress cell-cycle⁵⁰. Also found that breast tumors with high levels of P27 expression were rarely positive for Jab1/Csn5 expression and Jab1/Csn5 protein expressed levels were higher in oncogenically transformed breast cells and tumors than normal mammary epithelium. The function of Jab1/Csn5 is re-localizing P27 from nucleus to cytoplasm that inducing degradation P27 in cytoplasm by ubiquitin / proteasome pathway and finally, allowing breast tumor cells to progress into S phase⁵¹.

The results revealed a positive correlation ($p < 0.05$) between cyclin E and TNF- α in breast cancer patients. This correlation was interpreted by indirect effects of cyclin E on TNF- α and as shown from results of Dhillon, N.K. and Mudryj, M. that revealed that cyclin E over-expression cause decreasing of Bcl-2 protein levels in MCF-7 and T47D breast cancer cell lines⁵². The MCF-7 is the first cancer cell line isolated from Catherine Frances in Michigan, and called (MCF-7) Michigan Cancer Foundation and after 7 attempts to generate a monolayer from cancer cells⁵³. The Bcl-2 is over-expression in MCF-7 cell has suppress TNF- α and the response to TNF- α is increased in cyclin E over-expression cells and reported that an enhanced sensitivity of cyclin E over-expression cells to cytokine like TNF- α ⁵².

The negative correlation ($p < 0.05$) between HE 4 and cyclin E. Wang *et al*, interpreted the results on cell lines that HE 4 and ANXA 2 both localized in cell membrane and cytoplasm were over expressed and broadly interact in many malignant tumor cells, the cell lines that highly

expressed HE 4 and ANXA 2 considered have a greatest capability in migration, invasion, adhesion and proliferation and the interaction between HE 4 and ANXA 2 facilitate migration, adhesion, invasion and proliferation of malignant tumor cells⁵⁴. The ANXA 2 is Annexin 2 (ANXA 2) 36 – KDa calcium-dependent phospholipid binding protein in breast cancer, ANXA 2 participates in many cellular process including endocytosis, exocytosis of intracellular proteins, motility of cell, fibrinolysis and ion channel constitution⁵⁵. The ANXA 2 is related to the occurrence and development of many malignant tumor and play a central role in angiogenesis, migration, apoptosis, adhesion, invasion and proliferation of tumor cells⁵⁴.

The ANXA 2 over-expressed in many solid tumors like lung, colorectal, pancreatic, breast and hepatocellular cancer. Wu *et al*, explained that ANXA 2 significantly increase expression both P21 and P27, but decreased expression of CDK1, CDK2 and cyclin B⁵⁶. The cyclin E / CDK2 are responsible for G1 to S phase transition in cell cycle⁵⁷. So, from these finding can conclude that HE 4 by interaction with ANXA 2 can inhibit or decrease levels of cyclin E.

The hormonal receptors results clarified a positive (ER+/PR+) were 70 % with negative (ER-/PR-) 26 %. Several studies have indicated that patients with positive hormonal receptors have a significantly higher survival rate, since positive status have benefits from hormonal treatment, where this not demonstrated in negative side⁵⁸. Several studies have shown survival advantages of ER/PR receptors in breast cancer, it has been found that this survival advantages is substantially enhanced by adjuvant hormonal therapy which was given to patient with ER/PR positive tumor by reducing the recurrence rate during treatment period and reducing breast cancer mortality, therefore ER/PR expression status are regarded a good prognostic factor and predictor factor for response to endocrine therapy. The patients with ER+/PR+ tumor had superior survival compared with patients with ER-/PR- tumor and the estimated risk of death was significantly higher in patients with ER-/PR- tumor compared with patients with ER+/PR+ tumor⁵⁹.

The results of Her-2/neu demonstrated that Her-2/neu negative were 53 % more than positive 46 %. It is well known that overexpression of Her2 gene is a significant predictor of both short overall survival and time to relapse in breast cancer patient, Trastuzumab (Herceptin), which is monoclonal antibodies against extracellular domain of Her-2/neu, became the standard adjuvant treatment in breast cancer patients who appeared overexpression

of Her-2/neu. It has been proven that trastuzumab is an effective drug in improving survival for patients with early Her-2 positive breast cancer as well as metastatic Her-2 positive breast cancer⁶⁰.

In our study the cyclin E expression was 46 % phenotype III (+ nuclear and + cytoplasm) and 36 % phenotype II (+ nuclear and weak cytoplasm), patients with breast cancer whose tumors had no cytoplasmic cyclin E staining had an overall favorable prognosis, and those with any cytoplasmic cyclin E staining had a poor prognosis. We reported that LMW-E not full-length cyclin E, is most active in phosphorylating substrates and that LMW-E has a higher affinity than full-length cyclin E for binding CDK⁶¹.

LMW-E is more tumorigenic and patients whose tumors expressed LMW-E were shown to be at the highest risk for recurrence and death due to breast cancer. Because LMW-E lacks the nuclear localization of full-length cyclin E, the LMW-E accumulates in the cytoplasm, where it binds to CDK2 and retains kinase activity⁶². The expression of cytoplasmic cyclin E is related to the aggressiveness of the disease. The function of nuclear cyclin E has been attributed to cell-cycle progression, and overexpression of nuclear cyclin E leads to deregulation of cell proliferation, while cytoplasmic cyclin E has alternate functions that can affect signal transduction and metabolism⁶¹.

Conclusion:

The breast cancer is the common cancer that affected Iraqi women. The patients with higher serum of Ca 15-3, Ca 27.29 and elevated expression of ER, Her-2 and cyclin E levels are more likely to have breast cancer metastasis. So, both markers and by these correlations it has been demonstrated that they are as an indicator for helping clinicians to evaluate disease progression of breast cancer, predicting patient outcome and determining adjuvant treatment for better outcome.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for republication attached with the manuscript.
- The author has signed an animal welfare statement.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

Authors' contributions statement:

A.M., A.H. and H.L. contributed to the research design, collect the samples, implementation of the research, to the analysis of the results and to the writing of the manuscript

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العلاقة بين معلمات المصل و النسيج في المصابات العراقيات بسرطان الثدي

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الخلاصة :

يعد سرطان الثدي من أكثر الأورام الخبيثة انتشاراً بين النساء في جميع أنحاء العالم ، وفي العراق يحتل المرتبة الأولى والسبب الرئيسي لوفيات الإناث المرتبطة بالسرطان. صممت هذه الدراسة لبحث العلاقات بين معلمات المصل والنسيج من أجل توضيح دورها في تطور أو تراجع سرطان الثدي. معلمات الورم هي مجموعة من المواد ، تكون بشكل رئيسي ذات طبيعة بروتينية ، تنتج من الخلايا السرطانية أو من خلايا أخرى في الجسم استجابة للورم. أجريت الدراسة في الفترة من أبريل 2018 إلى أبريل 2019 بإجمالي عدد 60 امرأة مصابة بسرطان الثدي. تم جمع عينات الدم من النساء المصابات بسرطان الثدي في فترة ما بعد الجراحة وما قبل العلاج اللائي حضرن إلى مستشفى الأورام التعليمي في مدينة الطب في بغداد وتم تقييم معلمات المصل بتقنية ELISA وهي (Ca 15-3) Carbohydrate Antigen 15-3 و (Carbohydrate Antigen 29 Ca 27.29) ، هرمون مضاد مولر (AMH) ، عامل نخر الورم ألفا (TNF- α) ، إنترلوكين 6 (IL-6) ، إنترلوكين 10 (IL-10) وبروتين البربخ البشري 4 (HE4) . تم جمع عينات الأنسجة لنفس النساء المصابات بسرطان الثدي اللواتي حضرن إلى مدينة الطب ، بغداد بإجمالي عدد 30. تم تقييم معلمات الأنسجة باستخدام تقنية الكيمياء النسيجية المناعية وهي مستقبلات هرمون الاستروجين (ER) ، مستقبلات البروجسترون (PR) ، مستقبل عامل نمو البشرة البشري 2 (Her 2 / neu) و Cyclin E. أظهرت نتائج العلاقات بين معلمات المصل ومعلمات الأنسجة وجود ارتباط معنوي موجب (0.017) بين مستضد الكاربوهيدرات 27.29 ومستقبل عامل النمو البشرية البشري 2 ، (0.038) بين إنترلوكين-6 مع النمط الظاهري لسايكلين اي ، (0.051) بين عامل نخر الورم-الفا و شدة سايكلين اي ، (0.005) بين بروتين البربخ البشري-4 و مستقبل عامل النمو البشرية البشري 2 و ارتباط معنوي سالب (0.058) بين إنترلوكين-10 و مستقبل الاستروجين ، و (0.045) بين بروتين البربخ البشري-4 و شدة سايكلين اي. نستنتج من هذه العلاقات أن العلاقات الطردية تزيد من تطور المرض ، مثل العلاقة بين Ca 27.29 مع Her-2 / neu و cyclin E مع IL-6 و cyclin E مع TNF- α . وقد تساهم العلاقات العكسية في تأخر المرض ، مثل العلاقة بين IL-10 مع ER. من نتائج العلاقات في هذه الدراسة أتضح أن المعلمات Ca 27.29 ، Her-2 / neu ، cyclin E تلعب دوراً مهماً في تطور المرض.

الكلمات المفتاحية: هرمون مضاد مولر (AMH) ، سرطان الثدي ، Ca 15-3 ، مستقبل هرمون الاستروجين (ER) ، بروتين Epidiymis البشري 4 (HE4) ، عامل نخر الورم ألفا (TNF- α) .