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CD44 EXPRESSION AND ILGF I LEVEL IN DIABETIC AND NONDIABETIC PATIENTS WITH CANCER BREAST

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ABSTRACT

Breast cancer is the most common of all cancers worldwide, New cases of breast cancer diagnosed in 2012 were more than 1.7 million, it's responsible for nearly 700,000 deaths worldwide every year. The classical IHC assay is illustrated in (Figure-6) and involves detection of epitopes expressed within a tissue sample by targeting single protein-target using a "primary antibody" capable of binding those epitopes with high specificity.
After the epitope-antibody binding event, a "secondary antibody" capable of binding the primary antibody with high specificity is added. The secondary antibody is coupled to a reporter molecule. After the secondary antibody binding to primary antibody, a chemical substrate is added which reacts with the reporter molecule to produce a brown colored precipitate at the site of the whole epitope-antibody complex

INTRODUCTION

Breast cancer is the most common of all cancers worldwide, New cases of breast cancer diagnosed in 2012 were more than 1.7 million, it's responsible for nearly 700,000 deaths worldwide every year. (Ferlay et al., 2015).

Breast cancer is rare among men of all ages and women who are younger than 30 years. Incidence rates increase over a lifetime, slowing down around menopause (Jemal et al., 2007).

There is a very limited data as regard the prevalence and the incidence of breast cancer among Egyptian females, however, the National Cancer Institute (NCI) in Egypt reported that breast cancer represents 35.1% of all female cancers, (El Saghir et al., 2007).

In (**2009**), the NCI reported that the breast cancer account for 37.5% of all cancers affecting Egyptian females, (Shash, et al., 2010). Around 90% of these patients were diagnosed in late stages (III or IV) denoting unreliable screening programs (El Saghir et al., 2007).

Diabetes mellitus is a global public health concern. 382 million people had diabetes in 2013 and this number is expected to rise to 592 million by 2035, (Guariguata et al., 2013).

In Egypt Diabetes is a fast-growing health problem with a significant impact on morbidity, mortality, and health care resources. Currently, the prevalence of type II diabetes (T2D) in Egypt is around 15.6% of all adults aged 20 to 79 years, with an annual death of 86,478 related to diabetes, (Hagazi et al., 2015).

The International Diabetes Federation IDF, (2017) listed that Egypt is one of the 19 countries in the number of patients with diabetes. In 2017, the IDF estimated that 8,222,600 individuals have diabetes with Prevalence of diabetes in adults : 15.1% (The International Diabetes Federation, 2017).

Diabetes and breast cancer are quite prevalent chronic diseases among women. Approximately 16% of breast cancer patients suffered from diabetes, (Zhao & Ren, 2016).

Women with diabetes had a 23% greater risk of subsequent breast cancer than those without diabetes (De Bruijn et al., 2013)

In addition breast cancer patient who are diabetic have 32% increased risk of chemotherapy related complication and a 24-61% increased risk of all causes of mortality compared to breast cancer patients without diabetes (Srokowski et al., 2009)

Aim of the Work:

This study is aiming to assess the level of CD44 and ILGF 1 in cancer breast with & without type II diabetes mellitus

Patients & Methods

Fifty three Egyptian females had attended to Minia oncology center. All participants in our study were informed about the aim of this study and were given a written consent for their participation. Patients had the following inclusion and exclusion criteria Inclusion Criteria:

Inclusion Criteria

1. Egyptian female
2. Histologically confirmed breast cancer.
3. All stages of breast cancer will be included.

Exclusion Criteria:

1. Breast cancer patients that treated with chemotherapy or radiotherapy.
2. End organ failure such as heart, liver, or renal failure.
3. Hypertension.
4. Women with history or family history of any cancer.
5. Contraceptive pills.

This group subdivided into 2 subgroups according to diabetes status:

Group I: Type II diabetic patients with breast cancer.
Group II: Non-diabetic patients with breast cancer.

We used a comprehensive questionnaire to collect a complete history about menstrual, reproductive, menopausal and diabetes status as well as family history of breast cancer and other cancers.

Anthropometric Measurements and Clinical Examination:

All participants were subjected to the following measures:
The Height in centimeters & the Weight in kilograms.
The Body Mass Index (BMI) was calculated as the weight in kilograms divided by the square of height in meters.
Underweight: Less than 18.5
Normal weight: 18.5___24.9
Overweight: 25___29.5
Obese : 30__35
Morbid obesity: More than 35

The Waist circumference in centimeters measured by standard measure tape applied to waist at the level of anterior superior iliac spine.

Women with Waist circumference ≤88cm are considered normal
The Systolic and the diastolic blood pressure in Millimeter mercury (mmhg)

Laboratory Measurements:

The Blood samples were collected after an overnight fasting (about 8 hours). These samples were allowed to coagulate at room temperature for 10-20 minutes, then centrifuged for 5-minutes at the speed of 2000-3000 r.p.m. then the supernatant was removed, and plasma was separated and stored at – 20C° until the following tests were conducted:

Fasting blood glucose level in mg/dl will be determined by a glucose oxidase assay

Renal function tests,
Liver functions tests,
Complete blood counts,

The Diagnosis of Type II Diabetes Mellitus:

The diagnosis of Diabetes Mellitus was defined according to ADA, 2018 guidelines as any patient fill full one or more of the follow criteria:
FPG ≥ 126 mg/dl (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.* **(OR)**

2-h PG ≥ 200 mg/dl (11.1 mmol/L) during an OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.* **(OR)**

Hb A1C ≥ 6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.* **(OR)**

In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥ 200 mg/dl (11.1 mmol/L).

*In the absence of unequivocal hyperglycemia, results were confirmed by repeat tesing.

The Diagnosis Of Breast Cancer

The diagnosis of breast cancer was confirmed by multiple breast tissue biopsies, while the staging of breast cancer was determined based on the TNM system based on definitions and recommendations of European Society of Medical Oncology (ESMO), 2015, (**Senkus et al., 2015).**

The Immunohistochemistry Assay

Multiple breast tissue biopsies obtained from malignant breast tissues and adjacent normal tissues then fixed and stained with Immunohistochemical stain to assess:

Breast Tissue Estrogen Receptors expression.
Breast Tissue Progesterone Receptors expression.
Breast Tissue CD44 expression.
Breast Tissue IGF-1 receptors expression.

The basic principle of immunohistochemistry staining

The classical IHC assay is illustrated in (Figure-6) and involves detection of epitopes expressed within a tissue sample by targeting single protein-target using a "primary antibody" capable of binding those epitopes with high specificity.

After the epitope-antibody binding event, a "secondary antibody" capable of binding the primary antibody with high specificity is added. The secondary antibody is coupled to a reporter molecule

After the secondary antibody binding to primary antibody, a chemical substrate is added which reacts with the reporter molecule to produce a brown colored precipitate at the site of the whole epitope-antibody complex. **(Gremel, et al., 2014)**

Collection of Tissue Samples

Tissue samples were obtained from 53 women who underwent total mastectomy and lymph node dissection for primary breast cancer.

In-group I (breast cancer group) we collected tissue samples from malignant lesions and adjacent normal breast tissues including both breast epithelium and breast adipose tissues, while in group II (control group) we collected samples from adjacent normal breast tissues.

Preparation of Tissue Samples

Immediately after excision, tissue samples were fixed in 10% buffered formaldehyde solution; the purpose of formalin fixation is to produce chemical cross-linking of proteins within the tissue. This terminates all cellular processes and freezes the cellular components at the place and in the conformation; they were in at the time of fixation and prevent their degradation.

After adequate fixation, the tissue is further processed and ultimately embedded in paraffin blocks, which are then sectioned into thin slices (usually 4-10µm) using a micro-tome. These sections were transferred to glass slides for further processing.

Histopathological examination of these sections was based on the WHO classification of breast cancers.'

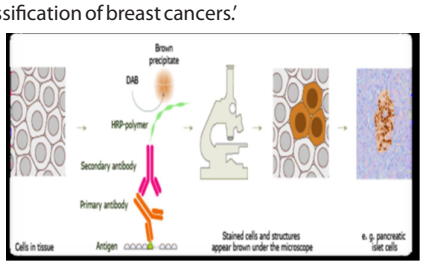


Figure 6: The basic principle of immunohistochemistry. (Gremel, et al., 2014)

In the schematic illustration (Figure 6) a formalin-fixed paraffin embedded tissue section is stained using a primary antibody directed towards a specific protein target. A solution containing the primary antibody is added to the tissue section and the antibodies are allowed some time to find and bind to their target. After this step, unbound and surplus antibodies are washed away and the secondary antibody is added. The secondary antibody, which carries a linker molecule with horseradish peroxidase (HRP) enzymes, is also allowed some time to bind to the primary antibody, followed by another washing step. After this, 3, 3' Diaminobenzidine (DAB) is added. The HRP enzyme transforms the DAB substrate into a

brownish precipitate that is deposited in the tissue at the site of the reaction, thus producing a visual representation of where the primary antibody first bound its target.

The immunohistochemical staining of CD44, IGF-1, ER and PR expression was carried out using 5-µm consecutive tissue sections obtained from breast tissue samples.

These sections were de-waxed in xylene and rehydrated in graded alcohols, then a formalin-fixed paraffin embedded breast tissue sections is stained using a primary anti-bodies targeting the following markers;

Insulin like growth factor -1 (IGF-1) Receptors: using rabbit polyclonal antibodies, manufactured by "Bioassay Technology Laboratory; Shanghai Korain Biotech Co., LTD", after dilution 1:100.

CD44, using rabbit polyclonal antibody, manufactured by "ProteinTech Group", after dilution 1:200.

Estrogen Receptors, using mouse monoclonal antibody F-10manufactured by "Santa Cruz Biotechnology", after dilution 1:200; based on recommendations of European Society of Medical Oncology (ESMO), (Senkus, et al., 2015).

Progesterone Receptors, using rabbit polyclonal antibody H-150 manufactured by "Santa Cruz Biotechnology" after dilution 1:100; based on recommendations of European Society of Medical Oncology (ESMO), (Senkus, et al., 2015).

HER2 receptors, mouse monoclonal antibody, manufactured by "Santa Cruz Biotechnology", after dilution 1:100; based on recommendations of European Society of Medical Oncology (ESMO), (Senkus, et al., 2015).

A solution containing the primary antibody was added to these tissue sections and the antibodies were allowed some time to find and bind to their targets. After that, the streptavidin-biotin-peroxidase complex (ABC Staining System, Santa Cruz Biotechnology) was applied to slides in order to reveal antibody-antigen reactions.

Then the slides embedded in a solution containing the secondary antibodies which linked to horseradish peroxidase enzyme that is capable of converting 3, 3' di-amino-benzidine (the coloring reagent which added later on) into brown precipitates that are de-posited in the tissues at the site of the antigen antibodies reactions. This brown stain was examined under light-microscopy easily and interpreted using a four-point scale (0, 1+, 2+ and 3+) depending on the percentage of tissues that trapped the brawn stain.

Interpretation of Immunohistochemistry Staining

The expression of CD44, IGF-1, HER2, PR, ER was analyzed by light microscopy in 10 different section fields, and the mean percentage of tumor cells displaying positive staining was scored as follow;

The expression of CD44, and IGF-1, in malignant tissues was categorized using the following four-point scale;

0 pointed to negative or <10% positive cells;
1+ pointed to 10 to 50% positive cells with weak brawn staining;
2+ pointed to >50% positive cells with weak brawn staining;
3+ pointed to >50% positive cells with strong brawn staining.

According to the final staining score for the expression of CD44: IHC 0 and 1+ were considered low expression and IHC 2+ and 3+ were considered high expression

The expression of ER-α and ER-β were classified as follows:
0 pointed to <10% positive cells;
1+ pointed to 10 to 50% positive cells with weak staining;
2+ pointed to>50% to 80 % positive cells with strong staining;
3+ pointed to > 80 % positive cells with strong staining.

All slides were counter-stained with H & E stain that discolors cellular cytoplasm with a pale bluish color, and stains cell nuclei in a darker bluish nuance.

Statistical Analysis

Data was analyzed with the IBM Statistical Package for the Social Sciences SPSS version 20.0. Descriptive data were given as mean ± standard deviation (SD).

Comparisons between the two groups were performed with t-test and covariance analysis, whereas comparisons among subgroups were performed using ANOVA test.

The Pearson correlation coefficients (r) were applied to measure the correlation for continuous normally and non-normally distributed variables, respectively.

The relationships clinical and pathological characters of Breast Cancer cases (TNM, tumor size, LN, and histological grade) and variables were analyzed by linear correlation and stepwise regression analysis.

The logistic multivariant regression analysis was used to analyze the relationships between adipocytokines, metabolic factors, and risk of LN metastasis of Breast Cancer.

P values less than 0.05 were considered statistically significant.

This study included 53 Egyptian females with median age was 50 (ranged from 35 to 70) years old with biopsy confirmed with breast cancer, sub- grouped into two groups

- Group-I: included 28 non-diabetic Egyptian females with breast cancer.
- Group-II: 25 Egyptian females with breast cancer and type II diabetes mellitus.

Immunohistochemistry was assessed for ER, PR, IGF 1R and Cd44
The results demonstrated in tables below as following:

Table (2): Shows that diabetic patients presented with significantly older age (P = 0.009), higher BMI (P = 0.003), higher fasting blood sugar (P = 0.001), larger tumor size (P = 0.009), and greater number of L.N metastasizes (P = 0.006) higher probability for distant Metastasizes (p= 0.003) compared with non-diabetic counterparts.

Table (3): Shows that diabetic patients significantly produce lesser ER & PR Expression in malignant tissues (p-value= 0.045 & 0.001 respectively) with significantly higher histological grading (p-value= 0.02) compared with non-diabetic patients.

Table (4): Shows significant increases in IGF-1R expression in malignant breast tissues compared with normal breast tissues (p = 0.001).

Table (5): Shows that IGF-1R Expression in malignant tissues were significantly higher in diabetic versus non-diabetic patients (p= 0.03), but there is no difference between diabetic and non-diabetic groups as regards IGF-1R Expression in normal tissues.

Table (6): Shows that CD44 Expression in malignant tissues were significantly higher versus normal tissue (p= 0.006).

Table (7): Shows that CD44 Expression in malignant tissues were significantly higher in diabetic versus non-diabetic patients (p= 0.028), also CD44 Expression in normal adipose tissues were significantly higher in diabetic versus non-diabetic groups (p=0.041).

Table (8) Shows significant Correlations between CD44 & IGF-1R in both malignant and normal breast tissues (p = <0.001, 0.008, <0.001 & 0.004 respectively) among both diabetic and non-diabetic patients.

Table (9): shows that IGF-1R expression in malignant breast tissues was negatively correlated with ER & PR expression in malignant breast tissues (p = 0.02 & r = -0.337) & (p = 0.01 & r = - 0.268) respectively, while it was positively correlated with tumor size (p = 0.015 & r = 0.332) and probability of distant cancer metastasis (p = 0.01 & r = 0.272). However, IGF-1R expression was not correlated with breast cancer grading (p=0.81, r=0.03) and L.N Metastasis (p=0.799, r=0.036).

Also ,Table (9): shows that CD44 expression in malignant breast tissues was negatively correlated with ER & PR expression in malignant breast tissues (p = 0.004 & r = - 0.387) & (p = 0.016 & r = - 0.331) respectively, while it was positively correlated with breast cancer grading (p = 0.004 & r = - 0.391) , tumor size (p = 0.031 & r = 0.297) L.N Metastasis (p=0.017, r=0.326)., and probability of distant cancer metastasis (p = 0.01 & r = 0.349).

The demographic and clinical data of studied groups were shown in

Table (2)Table (2): Demographic & clinical data of diabetic & non-diabetic group of patients:

Statistics#	D.M	N	Mean	Std. D	P-VALUE
Age (years)	Non-Diabetic	28	47.79	13.737	0.009*
	Diabetic	25	56.28	7.662	
BMI (kg/m2)	Non-Diabetic	28	28.652	5.1228	0.003*
	Diabetic	25	32.537	3.8118	
F.B.S (mg/dl)	Non-Diabetic	28	95.39	12.218	0.001*
	Diabetic	25	215.96	49.302	
Tumor size (cm2) (tumor surface area)	Non-Diabetic	28	8.321	6.6950	0.008*
	Diabetic	25	13.132	7.3809	
L.N Metastasis (No. of lymph nodes)	Non-Diabetic	28	10.57	10.189	0.006*
	Diabetic	25	18.84	9.254	
Distant Metastasis (%)	Non-Diabetic	28	8	-	0.001*
	Diabetic	25	14	-	

Independent samples t- test. BMI: Body Mass Index, FBS: Fasting Blood Sugar, L.N: Lymph Nodes, Std. D: Standard Deviation * significant P-Value < 0.05

Table (2): Shows that diabetic patients presented with significantly older age (P = 0.009), higher BMI (P = 0.003), higher fasting blood sugar (P = 0.001), larger tumor size (P = 0.009), and greater number of L.N metastasizes (P = 0.006) higher probability for distant Metastasizes (p= 0.001) compared with non-diabetic counterparts.

Table (3) ER, PR Expression and tumor grading among Diabetic Versus Non-Diabetic Patients:

Statistics#		Non-Diabetic		Diabetic		P-value
		N	%	N	%	
ER Expression In breast tissues (%)	Negative Expression	3	10.7 %	12	48.0%	0.045*
	Positive Expression	25	89.3 %	13	52.0%	
PR Expression In breast tissues (%)	Negative Expression	8	28.6 %	23	92.0%	0.001*
	Positive Expression	20	71.4 %	10	40.0%	
Tumor histological grading	Grade-II	27	96.4%	16	64.0%	0.02*
	Grade-III (High Grade)	1	3.6%	9	36.0%	

ANOVA -test. ER: Estrogen Receptors, PR: Progesterone Receptors * significant P-Value < 0.05

Table (3): Shows that diabetic patients significantly produce lesser ER & PR Expression in malignant tissues (p-value= 0.045 & 0.001 respectively) with significantly higher histological grading (p-value= 0.02) compared with non-diabetic patients.

Table (4) Expression of IGF-1R in Normal & malignant breast tissues

Statistics#	Tissues (a)	Mean + SD	Mean Rank	Sum of Ranks	Z	P-Value
IGF.1R Distribution (%) (% of positive cells throughout the slide	Normal	8.87 + 14.6	30.82	1633.50	- 7.7	0.001
	Malignant	62.4 + 31.4	76.18	4037.50		

Mann-Whitney test
* Significant P-Value < 0.05 (a): Grouping Variable are Normal vs. malignant tissues

Table (4): Shows significant increases in IGF-1R expression in malignant breast tissues compared with normal breast tissues (p = 0.001).

Table (5): Expression of IGF 1R among Diabetic & Non-Diabetic Patients with breast cancer:

Statistics#	Diabetes Status	N	Mean	Std. D	P-Value
IGF.1R Distribution (%) (Normal Tissues)	Non-Diabetic	28	6.07	10.6	0.24
	Diabetic	25	12.0	17.7	
IGF.1R Distribution (%) (Malignant Tissues)	Non-Diabetic	28	49.1	34.1	0.03
	Diabetic	25	66.2	28.2	

Independent samples t- test, Std. D: Standard Deviation * Significant P-value < 0.05 IHC: Immmunohistochemical staining

Table (5): Shows that IGF-1R Expression in malignant tissues were significantly higher in diabetic versus non-diabetic patients (p= 0.03), but there is no difference between diabetic and non-diabetic groups as regards IGF-1R Expression in normal tissues.

Table (6) Expression of CD44 among Normal & malignant breast tissues

	Expression	Normal	Malignant	P value
		N=53	N=53	
CD 44	Low	35(66%)	21(39.6%)	0.006*
	High	18(34%)	32(60.4%)	

- groups- Chi square test for qualitative data between the two
*: Significant level taken at P value < 0.05

Table (6): Shows that CD44 Expression in malignant tissues were significantly higher versus normal tissue (p= 0.006),

Table (7) Expression of CD44 findings among Diabetic & Non-Diabetic Patients

Statistics#	Diabetes Status	Expression	Number %	p-value
Cd44 expression in normal tissue	Non-Diabetic (N=28)	Low	22(78.6%)	0.041*
		High	6(21.4%)	
	Diabetic (N=25)	Low	13(52%)	
		High	12(48%)	

Cd44 expression in malignant tissue	Non-Diabetic (N=28)	Low	15(53.6%	0.028*
		High	13(46.4%)	
	Diabetic (N=25)	Low	6(24%)	
		High	19(76%)	
* significant P-Value < 0.05 Chi square test for qualitative data between the two groups				

Table (7): Shows that CD44 Expression in malignant tissues were significantly higher in diabetic versus non-diabetic patients (p= 0.028), also CD44 Expression in normal adipose tissues were significantly higher in diabetic versus non-diabetic groups (p=0.041).

Table (8): Correlations of CD44 expression with IGF-1R expression

Group	DM	IGF with CD 44	
		R	P value
Normal	No	0.556	0.004*
	Yes	0.944	<0.001*
Malignant	No	0.520	0.008*
	Yes	0.806	<0.001*

Table (8) Shows significant Correlations between CD44 & IGF-1R in both malignant and normal breast tissues (p = <0.001, 0.008, <0.001 & 0.004 respectively) among both diabetic and non-diabetic patients

Table (9): Correlation of CD44& IGF-1R expression with pathological findings:

correlation		IGF-1R(malignant tissues)	CD44(malignant tissues)
ER Expression	(r)	- 0.337*	-0.387
	p-value	0.02	0.004*
PR Expression	(r)	- 0.268*	-0.331
	p-value	0.01	0.016*
Tumor Grading	(r)	0.03	0.391
	p-value	0.81	0.004*
Tumor size	(r)	0.332	0.297
	p-value	0.015	0.031
L.N Metastasis (No. of lymph nodes)	(r)	0.036	0.326
	p-value	0.799	0.017
Distant Metastasis	(r)	0.272*	0.349
	p-value	0.01	0.010*

Spearman's correlation * Significant P-value < 0.05 PR: Progesterone receptors ER:
Estrogen receptors ®: Correlation coefficient

Figure 7 :IHC of CD44 in Normal and Malignant Tissue
Membranous staining brawny stain

Figure A CD44 –ve in normal adipose tissue

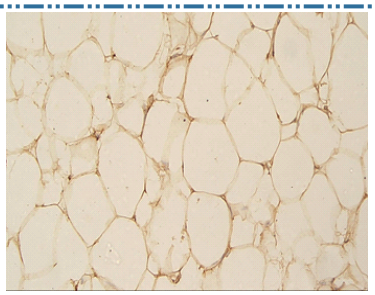


Figure B CD44 +ve in normal adipose tissue

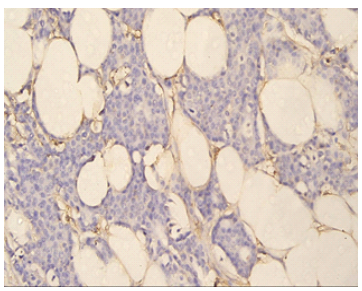


Figure C CD44 -ve in malignant tissue

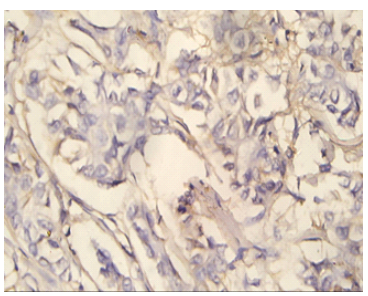
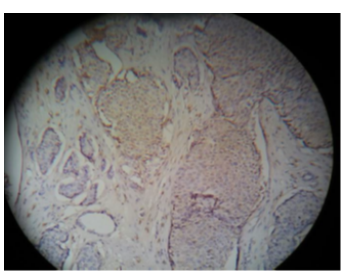
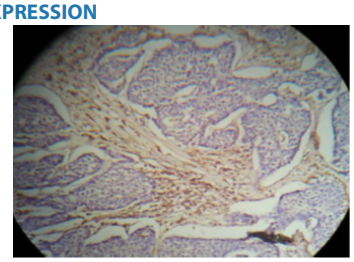


Figure D CD44 +ve in malignant tissue

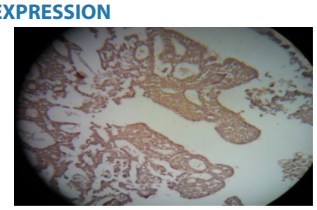
Figure 8: IHC of IGF-1R (membranous staining) in malignant tissue



(+) IGF-1R EXPRESSION



(++) IGF-1R EXPRESSION



(+++) IGF-1R EXPRESSION

DISCUSSION

Breast cancers are the most common cancers affecting females worldwide, Moreover, breast cancers are responsible for nearly 700,000 deaths worldwide every year, (Ferlay et al., 2013).

Type II diabetes mellitus and breast cancers are major causes of morbidity and mortality worldwide, (xiao et al., 2016).

Type II diabetes mellitus has a 20% increased risk for breast cancer incidence and increase cancer mortality with breast cancer from 15% to 30%, (Tsilidis et al., 2015).

Approximately 16% of breast cancer patients suffered from diabetes, (Wolf, et al., 2011). Diabetes mellitus has been identified as an independent predictor of poor prognosis of breast cancers, (xiao et al., 2016).

This study was aiming to clarify the associations of Type II DM, CD44 and IGF 1R expression with clinicopathological presentations of breast cancer among sample of Egyptian females.

This sample of Egyptian diabetic females with breast cancers had older age (mean=56.2±7.6 vs 47.79±13.7, P: 0.009), higher BMI (mean= 32.5±3.8 vs 28.65±5.1, P: 0.003) compared with non-diabetic patients.

These results are in consistence with Juhue Luo et al., 2014 who showed that women with diabetes with cancer breast were significantly older, (mean= 71.9, P<0.0001) compared with nondiabetic patients cancer breast.

These results also are in consistence with He et al., 2015 who showed that Patients with Type II DM with Breast Cancer were older (P<0.001) and the postmenopausal proportion was higher (67.9 vs. 42.0%, P<0.001). Patients with Type II DM were likely to be overweight or obese (BMI, ≥25 kg/m2, P<0.001) Compared with nondiabetic patients.

Older adults are at high risk for the development of type II diabetes due to the combined effects of increasing insulin resistance and impaired pancreatic islet function with aging. Age-related insulin resistance appears to be primarily associated with adiposity, sarcopenia, and physical inactivity, (Amati et al., 2009).

Also, The current study showed that the diabetic females with breast cancers are presented with higher breast cancer TNM scoring, larger tumor size (p: 0.03) greater number of L.N metastases (p: 0.02), and higher probability for distant metastasis (p: 0.01) when compared with nondiabetic patients.

In addition, this study showed that this sample of Egyptian diabetic females with breast cancer are more frequently presented with more aggressive breast cancer with significantly higher tumor grades (p :0.02) higher chance for occurrence of ER negative (p :0.045), and PR negative breast cancer (p : 0.001) when compared with non-diabetic patients.

Our results are in consistence with He et al., 2015 who showed that Type II DM with Breast Cancer had higher TNM staging (P=0.001), higher Lymph nodes involvement (P=0.001) compared with nondiabetic patients ,conversely, he found no statistical difference in tumor size (p = 0.052).

This study also in consistency with Li et al., 2011, who showed that majority of histological grade in diabetic patients with breast cancer, was II + III-class, Lymph node metastasis was more in breast cancer patients in the diabetic group.

In addition, Srokowski et al., 2009 demonstrated that a higher percentage of women had Type II DM with Breast Cancer presented with a more advanced stage than their nondiabetic counterparts (P < .0001).

Similarly , Zhihua et al, 2011 and Larsson et al., 2007 confirmed that patients with breast cancer and type II diabetes mellitus has been associated with adverse outcomes throughout the full course of disease i.e., initial presentation, treatment, recurrence patterns, and cancer related mortality.

These data reflect the poor prognostic effect of type II diabetes mellitus on outcomes of breast cancers.

Lorincz et al., 2006 have proposed several hypotheses to explain the association between type II D.M and postmenopausal breast cancers, such as :The metabolic syndrome, results in hyperinsulinemia with relatively overexpression of insulin receptors and insulin-like growth factor (IGF-1), which act as mitogens, The underlying obesity (most type II D.M patients are actually obese) which results in hyperestrogenemia via peripheral aromatization of androgens in adipose tissue and Adipokines such as leptin, chemerin and Adiponectin with their autocrine, paracrine, and endocrine roles that associated with initiation and progression of breast cancer.

We are in disagreement with Luo et al., 2015 who found that There were no statistically significant differences in tumor characteristics, including tumor size, lymph nodes, grade, estrogen and progesterone receptor status compared with nondiabetic patients. These results may be explained as these studies did not adjust for some confounding factors such as duration of D.M, the use of insulin versus other oral anti-diabetic medication and intensive glycemic control versus less controlled diabetes which must be taken into account to analyze the impact of diabetes on breast cancer prognosis.

IGF1 is the major mediator of the effects of the growth hormone; it thus has a strong influence on cell proliferation and differentiation and is a potent inhibitor of apoptosis. (Fürstenberger et al., 2002).

IGF-I stimulation contributes to breast cancers progression via its mitogenic and anti¬apoptotic effects on the mammary epithelial cells and additionally protects breast cancers cells from the toxic effects of radio- and chemotherapy (Hankinson et al., 2008).

In our study, we found that there was significant increase in the expression of IGF-1R in malignant breast tissues com¬pared with normal breast tissues, (mean: 62.4 %± 31.4 vs 8.87% ±14.6, p: 0.01).

Also, malignant breast tissues in diabetic patients significantly express more IGF-1 re¬ceptors compared with non-diabetic patients, (mean: 66.2%±28.2 vs 49.1%±34.1 p: 0.03).

We are in agreement with Xin et al., 2015 who found that IGF 1 receptor expression in type II DM with breast cancers was higher compared with nondiabetic patients (P : 0.044).

Also, Yerushami et al 2012 showed that IGF-1R expression was higher in malignant cases compared to the benign cases (46 vs 15%).

Similarly, Chong et al., 2006 found that IGF-1R expression was higher in tumor tissue compared to Adjacent Non-Cancerous Tissues (P=0.038).

Our study explored that IGF-1 receptors expression was significantly associated with higher breast cancer TNM staging; larger tumor size (P: 0.004), higher rate of L.N metastasis (P: 0.006), higher incidence of distant metastasis (P < 0.001).

Also, IGF-1R expression was significantly correlated with higher incidence of ER negative (P: 0.02) and/or PR negative breast cancers (P: 0.01). However, IGF-1R expression was not correlated with breast

cancer grading (p=0.81).

We are in agreement with Bahhnassy et al.,2015 who found that high levels of IGF-I were significantly associated with lymph nodes metastasis (P: 0.007), distant metastasis (P<0.001) ,higher incidence of ER negative and PR negative breast cancers (P<0.001).

Also, Al Sarakbi et al 2006 showed that the level of IGF-I mRNA expression seems to correlate with the nodal status which is the best single prognostic indicator in human breast cancer.

We are in agreement with Yue et al., 2015 who identified that IGF-1R overexpression as a marker of an aggressive breast cancers phenotype. Also, they concluded that high levels of IGF-1R were identified as independent predic¬tors for poor overall survival and high rate of lymph nodes invasion

Also, this study was in agreement with pollak et al., 2004 who found that IGF-IR is often ex¬pressed in breast cancers, and their overexpression has been associated with worse progno¬sis.

Once more, Yang et al., 2013 concluded that inhibition of IGF-1R has been viewed as a potentially valuable target for breast cancers treatment.

These results signify that overexpression of IGF-1 receptors in malignant tissues con¬veys poorer prognosis in patients with breast cancers especially with type II diabetes mellitus.

Many epidemiological studies have suggested that insulin and IGF-1 play an important role in the regulation of cancer. The increased insulin or IGF-1 level, which presents in Type II DM and obesity, is strongly associated with increased cancer risk and mortality, play an important role as mitogens (Gu et al., 2013).

In contrast , Xin et al .,2015 demonstrated that there was no correlation between IGF1R expression and other clinicopathological factors, such as size of tumor, lymph node (LN) metastasis, pathological type, ER and PR status. (p= o.221, 0.615, 0.301, 0.805, 0.257, respectively) in diabetic patient.

Also, Shin et al., 2014 showed that Positive IGF-1R expression was associated with a positive hormone receptor status (for both ER and PR) (p<0.001). In addition, IGF-1R positivity was associated with low histological grades (p<0.001). There was no correlation between IGF-1R expression and, tumor size, and the presence or absence of lymph node metastasis (LN status).

Additionally, Yerushaltni et al., 2012 found that IGF-1R is highly expressed in patients with early Breast cancers and overall positively associated with good prognostic variables

The inconsistency of results between these studies and our study could be explained by the following:

IGF-1R may have different prognostic impact in different breast cancers subtypes; IGF-1R has been associated with favorable outcome in patients with the luminal B Breast cancers molecular subtype, in contrast to HER2 enriched patients, (Yerushalmi et al., 2012).

IGF-1R has been associated with favorable outcomes in patients with ER positive breast cancers compared with poor outcomes in patients with ER negative breast cancers.

IGF-1R has been associated with very poor outcomes in patients with triple negative breast cancers. High levels of IGF-IR were detected in 100% of the triple negative breast cancers, (Sarfstein et al., 2006)

Overwhelming number of studies have been carried out to explore the role of CD44 in cancer. As a well-known marker of Cancer Stem Cells, CD44 promotes carcinogenesis of diverse tumor types, including breast cancer (Xie, et al., 2012), pancreatic (Wang et al., 2014) and colorectal (Du et al., 2008).

CD44 is a marker of tumor-initiating cells, plays a role in tumorigenesis, and linked to the progression of breast cancer (Nam K, et al., 2015). CD44 was also reported to have an impact on the prognosis of breast cancer including recurrence (So JY, et al., 2013) and chemo resistance (Boulbes DR, et al., 2015).

Our results found that CD44 was significantly expressed in higher levels in malignant breast tissues compared with normal breast tissues (60.4%vs 34%) with p value (0.006)

In addition, malignant breast tissues in diabetic patients express higher levels of CD44 compared with non-diabetic patients (76%vs 46.4%) with p value (0.028)

We are in agreement with (Xu et al., 2016) who found that the level of CD44 was higher in breast cancer tissues than in normal breast tissues (p=0.034).

This study explored that CD44 receptors expression was positively correlated with higher breast cancer TNM staging; larger tumor size (p = 0.031) L.N Metastasis (p=0.017), and probability of distant cancer metastasis (p = 0.01).

Also, CD44 expression was significantly correlated with higher histological grades of breast cancer (P: 0.004), and higher incidence of ER negative (P: 0.004) and PR negative breast cancers (p: 0.016).

We are in agreement with Xu et al., 2016 who found that CD44 protein abundance was greatly elevated in high-grade breast cancer tissues (p: 0.005) and higher level of CD44 was significantly correlated with lower status of ER (p: 0.040) and PR (p: 0.023).

Also, We are in consistence with McFarlane et al., 2015 who showed that Strong CD44 expression associated with high-grade tumors (p: 0.046), progesterone receptor negative (p: 0.029) and estrogen receptor negative tumors (p: 0.001).

In addition, our study in consistence with Klingbeil et al., 2010 who reported that CD44 expression is particularly enriched in estrogen receptor (ER)-negative, progesterone receptor (PR)-negative and/or human epidermal growth factor receptor 2 (HER2)-negative breast cancers, which have the worst clinical prognosis and outcome.

Also, we are in agreement with Loi et al., 2006 who indicated that an increasing trend in CD44 expression with increasing histological grade of breast cancer, with axillary lymph nodes metastasis suggesting that CD44 may be linked to tumor differentiation and increasing aggressiveness.

In addition, Bankfalvi et al., 1998 observed that increased levels of CD44 expression were correlated with poor prognosis and metastatic involvement of the axillary lymph nodes in breast cancer. Several studies indicated that high CD44 expression is associated with poor prognosis and might be considered a target for therapy in breast cancer (McFarlane et al., 2015).

These results signify that overexpression of CD44 receptors in malignant tissues con¬veys poorer prognosis in patients with breast cancers in type II diabetes mellitus.

In contrast, we are in disagreement with Horiguchi et al., 2010,who found that higher CD44 expression was significantly correlated with smaller tumor size, negative axillary lymph node metastasis and lower stage ,ER and PR status (p=<0.0001 ,0.905 ,0.875 ,0.746 ,0.873 respectively).

Also ,we are in disagreement with Natkshatri et al., 2009 who demonstrated that CD44 often correlates with a favorable prognosis in early noninvasive breast cancer, and indeed, CD44 may not function as a marker of tumor-initiating cells at this phase in breast cancer progression.

This inconsistency of results between these studies and our study could be explained by the variations in assessing CD44 mRNA expression might also contribute to heterogeneity. Methods and cutoff values used to assess CD44 expression were different, (Xu et al., 2016).

This result, Shows significant positive Correlations between CD44 & IGF-1R in both malignant and normal breast tissues (p = <0.001, 0.008, <0.001 & 0.004 respectively) among both diabetic and non-diabetic patients.

Summary

This study included 53 Egyptian females who attended to Minia oncology center, with median age 50 (ranged from 35 to 70) years old with biopsy confirmed breast cancer, they sub- grouped into two groups:

- Group-I: included 28 non-diabetic Egyptian females with breast cancer.
- Group-II: 25 Egyptian females with breast cancer and type II diabetes mellitus.

The aim of this study was to evaluate the level of CD44 and ILGF 1 in cancer breast with & without type II diabetes mellitus and to correlate the level of them with the clinicopathological features with cancer breast with and without diabetes mellitus

All participants included in our study were subjected to careful history taking, Anthropometric measurements (Height ,Weight, BMI and waist circumference) and full clinical examination, laboratory studies included (fasting blood sugar, renal function tests, Complete blood counts and liver function tests),

The immunohistochemical staining of CD44, IGF-1, ER and PR expression was carried out in Multiple breast tissue biopsies obtained from malignant breast tissues and adjacent normal tissues .

Diabetic patients presented with significantly older age (P = 0.009), higher BMI (P = 0.003), higher fasting blood sugar (P = 0.001), larger tumor size (P = 0.009), and greater number of L.N metastasizes (P = 0.006) higher probability for distant Metastasizes (p= 0.003) compared with non-diabetic counterparts.

Diabetic patients significantly produce lesser ER & PR Expression in malignant tissues (p-value= 0.045 & 0.001 respectively) with significantly higher histological grading (p-value= 0.02) compared with non-diabetic patients.

IGF-1R expression in malignant breast tissues compared with normal breast tissues (p = 0.001).

IGF-1R Expression in malignant tissues were significantly higher in diabetic versus non-diabetic patients (p= 0.03).

CD44 Expression in malignant tissues were significantly higher versus normal tissue (p= 0.006).

CD44 Expression in malignant tissues were significantly higher in diabetic versus non-diabetic patients (p= 0.028), also CD44 Expression in normal adipose tissues were significantly higher in diabetic versus non-diabetic groups (p=0.041).

Positive Correlations between CD44 & IGF-1R in both malignant and normal breast tissues (p = <0.001, 0.008, <0.001 & 0.004 respectively) among both diabetic and non-diabetic patients.

IGF-1R expression in malignant breast tissues was negatively correlated with ER & PR expression in malignant breast tissues (p = 0.02 & r = -0.337) & (p = 0.01 & r = - 0.268) respectively, while it was positively correlated with tumor size (p = 0.015 & r = 0.332) and probability of distant cancer metastasis (p = 0.01 & r = 0.272). However, IGF-1R expression was not correlated with breast cancer grading (p=0.81, r=0.03) and L.N Metastasis (p=0.799, r=0.036).

Cd44 expression in malignant breast tissues was negatively correlated with ER & PR expression in malignant breast tissues (p = 0.004 & r = - 0.387) & (p = 0.016 & r = - 0.331) respectively, while it was positively correlated with breast cancer grading (p = 0.004 & r = - 0.391) , tumor size (p = 0.031 & r = 0.297) L.N Metastasis (p=0.017, r=0.326)., and probability of distant cancer metastasis (p = 0.01 & r = 0.349).

CONCLUSIONS:

1) Type II diabetes mellitus with its multiple risk factors, appears to be an important contributors of breast cancers risk among Egyptian females.

2) The presence of Type II diabetes mellitus delivers poorer prognostic effects on breast cancers among Egyptian females.

3) 1GF-1R over-expression was a risk factor for occurrence and progression of breast cancers among Egyptian females

4) CD 44 over-expression was a risk factor for occurrence and progression of breast cancers among Egyptian females.

4) Both IGF-1R and/or CD44 over-expression associated with poor clinicopathological outcomes of breast cancers among diabetic females.

Recommendation and limitation of the study

OUR STUDY RECOMMENDED THAT:

1. Women with Type II diabetes should seek regular preventive screenings of breast cancers, such as clinical breast examinations and mammograms.

2. Identification of pathways that are link type II D.M, metabolic syndrome and breast cancers may offer new targets for early detection and therapy of breast cancers.

3. Many more studies are needed to determine the feasibility of drugs that targeting IGF1R and CD44 in reducing insulin-mediated growth and proliferation of cancer cells

LIMITATIONS OF THIS STUDY:

1. First, our findings were based on tissue findings and IHC only, it was better to reinforce this work with serum assay of IGF-1, CD44, and insulin level applied to larger number of patients for better correlation between tissues and serum findings.

2. Further multicenter researches with longer follow-up interval are needed to assess the impact of diabetes on survival outcomes among Egyptian females with breast cancers.

3. Finally, this study did not adjust for some confounding factors such as duration of D.M, the use of insulin versus other oral anti-diabetic medication and intensive glycemic control was not taken into account to analyze the impact of diabetes on breast cancer prognosis.

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