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AnnexinA3 as a potential breast cancer diagnostic marker

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Abstract

Background: Research has demonstrated that Annexin A3 is significantly elevated in breast cancer (BC) patients in comparison to healthy individuals and is negatively associated with general survival and prognosis.
Aim and objectives: To explain the possible function of Annexin A3 in the development and metastasis of BC.
Patients & methods: The present research involved 90 female cases that were chosen from the South Egypt Cancer Institute, Surgery Department, and Assiut University during the period from March 2019 to December 2019. They were categorized into 3 groups as follows: 15 patients with benign breast diseases, 55 carcinoma patients, and 20 control groups
Results: AnnexinA3 had a sensitivity of 84.13% and a specificity of 66.53% in the diagnosis of BC cases. AnnexinA3 showed a significant relationship with TNM stage, distant metastasis, and the progesterone receptor. According to the relative gene expression of annexin A3, a highly statistically significant variance was observed among the control, benign, and carcinoma groups. According to clinicopathological features of the BC cases, the majority of patients had grade II (74.54%), the majority of patients had T3 tumor (41.81%), 22 cases (40%) had N2 regional LN, 47 cases (85.45%) had no distant metastasis, 27 cases (49.09%) had stage III, 36 cases (65.45%) had a positive estrogen receptor, 29 cases (52.72%) had a positive progesterone receptor and 27 cases (49.09%) had a positive HER2\ neu.
Conclusion: We concluded that AnnexinA3 is a reliable diagnostic marker for breast cancer. Further prospective investigations with larger scales are needed to confirm our results.

Introduction

BC, the most frequent cancer in females, is primarily caused by metastasis, and despite advancements in diagnosis and treatment, traditional treatments are limited by tumour cell resistance, necessitating ongoing research for new therapeutic approaches. [1]

Annexin A3 (ANXA3), a calcium-dependent phospholipid binding protein, plays a major role in the signal transduction of cell proliferation, apoptosis, and differentiation, as well as inflammation, tumor development, and occurrence. According to research, ANXA3 is significantly elevated in breast cancer cases in comparison to healthy individuals & negatively associated with overall survival and prognosis **[2].**

ANXA3 has been related to a variety of malignant neoplasms, like BC, prostate cancer, nasopharyngeal carcinoma, colorectal cancer, pancreatic cancer, hepatocellular carcinoma, thyroid cancer, renal cancer, gastric cancer, osteosarcoma, & lung cancer **[3].**

ANXA3, which is also known as placental anticoagulant protein a3 or lipocortin 3, is encoded on 4q13–q22. The molecular weight of the ANXA3 molecule is 36 kDa, which contains 323 amino acids, and thirty-three kilodaltons, which contains 284 amino acids. It has two isoforms **[4]**. While most neoplasms show only the thirty-six kilodaltons form, certain cells express both isoforms, or cells like myeloid cells, prostate adenocarcinoma cells, and rat brain cells might express only 1 of the 2 isoforms (thirty-three or thirty-six kilodaltons) **[5]**. The N-terminal region of ANXA is variable, while the C-terminal region is fixed. 4 or 8 annexin repeats comprise the C-terminal region. Phospholipid and Ca2+ binding sites are present in approximately 70 amino acids of

each annexin repeat. The N-terminal region of annexins, which is responsible for a variety of biological activities & functions, is composed of 20–200 amino acids. An N-terminus, four additional repeat domains, & a C-terminus are all present in ANXA3. Compared to the thirty-six kilodaltons isoform, the thirty-three kilodaltons isoform of ANXA3 lacks the 1ST 39 amino acid residues of the N-terminal region [6,7]. The work aimed to clarify the possible function of Annexin A3 in progress and the metastasis of BC.

Materials and methods

Patients : The current research involved 90 female cases that were chosen from the South Egypt Cancer Institute, Surgery Department, and Assiut University during the period from March 2019 to December 2019. They were categorized into 3 groups as follows: 15 patients with benign breast disease, 55 carcinoma patients, and 20 control groups

Ethical consideration: The Ethics Committee of the Faculty of Medicine, Assiut University, accepted the research protocol; each participant gave written informed consent before their participation in the investigation (Code: 17100825).

Exclusion criteria: cases with malignancies located somewhere else in the body, those who are receiving neoadjuvant chemotherapy, those who have chronic cardiac diseases, and those who have renal disease or neurodegenerative disease.

Methods

Method of determination of serum annexin A3 (ANXA3):

Serum annexin A3 levels (in ng/ml) were evaluated utilizing an ELISA kit (Cloud Clone Corporation, USA, Catalogue No. E-02676Hu) regarding the instructions of the manufacturer.

Test principle

The Sandwich-ELISA method was utilized in this ELISA kit. The micro-ELISA plate involved in this kit was pre-coated with an antibody that was definite for ANXA3. The specific antibody was combined with the appropriate micro-ELISA plate wells to add standards or samples. An Avidin-Horseradish Peroxidase (HRP) conjugate and a biotinylated detection antibody specific for ANXA3 are subsequently added to each microplate well & incubated. Free components were eliminated through the process of washing. Substrate solution has been introduced to each well. The only wells that exhibited a blue color were those that contained Avidin-HRP conjugate and ANXA3. The color changes to yellow upon the addition of stop solution, which terminates the enzyme-substrate reaction. The optical density (OD) has been determined through spectrophotometry at a wavelength of 450 nanometers. The concentration of ANXA3 was directly proportional to the OD value.

Reagent preparation: We diluted the 20× wash solution with deionized or distilled water. Prior to use, the reagents were brought to room temperature (18–25 °C) at a ratio of 1:20.

Assay procedure:

All samples and reagents have been brought to room temperature (18–25 °C). The samples were centrifuged once more prior to the assay, following their thawing. Before pipetting, the reagents were thoroughly mixed by gently swirling. Foaming was prevented. Duplicate analyses were conducted for all standards. First, fifty microliters of the standard were added to the standard well. Then, the testing sample is well diluted with forty microliters of sample diluent after adding 10 µl of the testing sample. The blank well didn't need any additions. Then, each well was covered with an adhesive strip, 100 µl of HRP-conjugate reagent has been added and it was incubated at thirty-seven degrees Celsius for sixty minutes. The process was repeated four times for a total of five washes, aspirating each well and washing it. A manifold dispenser, squirt bottle, or auto washer was used to fill each well with 400 µl of wash solution. Good performance necessitates the full removal of liquid at each stage. Any residual wash solution has been removed by aspirating or decanting following the final wash. The plate was blotted against clean paper towels after inverting it. Fifty microliters of chromogen solution A and 50 µl of chromogen solution B were added to each well and they were gently combined and incubated at 37 °C for fifteen minutes. Shield from light, then we added 50 µl of Stop Solution to each well. The color of the wells changed from blue to yellow. We gently tap the plate to ensure that the wells are thoroughly mixed if the color change isn't uniform or the color in the wells is green. Within 15 minutes, a microtiter plate reader has been utilized to determine the optical density (O.D.) at 450 nm.

Calculation of outcomes

The standard curve was utilized to identify the quantity of an unknown sample. It was produced by plotting the average optical density (450 nanometers) of each of the six standard concentrations on the vertical (Y) axis against the corresponding concentration on the horizontal (X) axis. The average optical density value was determined for each standard and sample. Before interpreting the results, the mean value of the zero standard was subtracted from all O.D. values. The standard curve was constructed using statistical software or graph paper**.**

Primarily, we located the optical density value on the Y-axis & extended a horizontal line to the standard curve to identify the quantity in each sample. Then, we draw a vertical line to the X axis at the intersection & read the corresponding concentration. The sample concentration has been evaluated using a standard curve. The diluted sample value was multiplied by the dilution factor.

Results & discussion:

Table (1): Distribution of general characteristics of all cases.

	Control N=20	Benign N=15	Carcinoma N=55	P-value
Age at diagnosis				
Mean± Standard Deviation	44.8±7.41	40.6±11.3	53.87±13.29	**P1: ≤0.001*** **P2: 0.559** **P3: 0.012*** **P4: ≤0.001***
Age at menarche (years)				
Mean± Standard Deviation	12.9±1.28	13.89±2.41	13.42±1.29	**P1: 0.163** **P2: 0.144** **P3: 0.395** **P4: 0.542**
Age at marriage (years)				
Mean± Standard Deviation	18.47±1.52	20.16±3.89	19.3±4.35	**P1: 0.434** **P2: 0.404** **P3: 0.685** **P4: 0.722**
Number of deliveries				
Mean± Standard Deviation	1.67±1.74	1.83±1.58	3.75±2.33	**P1: ≤0.001*** **P2: 0.973** **P3: ≤0.001*** **P4: 0.006***
Breast feeding duration in years				
Mean± SD	2.67±2.32	3.38±2.54	5.85±3.52	**P1: ≤0.001*** **P2: 0.786** **P3: ≤0.001*** **P4: 0.022***
Family history of breast cancer				
Yes	0 (0%)	3 (20%)	14 (25.45%)	**0.04***
No	20 (100%)	12 (80%)	41 (74.54%)	

P value >0.05: Not significant, P value <0.05 is statistically significant, p<0.001 is greatly significant., SD: standard deviation, P1: Comparison between **control** &**Carcinoma**& **benign**, P2: Comparison between **control**& **benign**, P3: Comparison between **control** &**Carcinoma**, P4: Comparison between **Carcinoma**& **benign**.

According to demographic data, table 1 demonstrates that there was statistically insignificant variance as regard to age at menarche (years) and age at marriage (years) and a statistically significant variance was detected as regard family history of BC while a highly statistically significant variance was detected as regard age at diagnosis, number of deliveries and breast-feeding duration in years between Control, Benign and Carcinoma groups.

Table (2): Distribution of pathological types of benign & malignant breast diseases.

	Benign N= 15		**Carcinoma N= 55**	
	No.	**%**	**No.**	**%**
Ductal carcinoma insitu	-	-	3	5.45
IDC	-	-	52	94.54
Fibroadenoma	12	80	-	-
Fibrocystic diseases	3	20	-	-

According to pathological types, table 2 shows that 3 cases (5.45%) had Ductal carcinoma in situ and other had IDC in Carcinoma group while 12 cases (80%) had Fibroadenoma and other had Fibrocystic diseases in Benign group.

Table (3): The clinic-pathological characteristics of the BC cases.

	Carcinoma N=55	
	Number of cases	**%**
Grade		
II	41	74.54
III	14	25.45
Tumor		
T1	12	21.81
T2	17	30.9
T3	23	41.81
T4	3	5.454
Regional LN		
N0	5	9.09
N1	21	38.18
N2	22	40
N3	7	12.72
Distant metastasis		
M0	47	85.45
M1	8	14.54
Stage		
I	2	3.63
II	20	36.36
III	27	49.09
IV	6	10.90
Estrogen receptor (ER)		
positive	36	65.45
negative	19	34.54
Progesterone receptor (PR)		

	Carcinoma N=55	
	Number of cases	**%**
positive	29	52.72
negative	26	47.27
HER2\ neu		
positive	27	49.09
negative	28	50.9
Side of breast affected		
RT	26	47.27
LT	29	52.72
Menopausal state		
Premenopausal	27	49.09
Postmenopausal	28	50.9
Molecular type		
Triple negative	14	25.45
Luminal A	15	27.27
Luminal B	23	41.81
HER2 enriched	3	5.45

According to clinicopathological characteristics of the breast cancer patients, table 3 shows that most of patients had grade II (74.54%), majority of patients had T3 tumor (41.81%), 22 cases (40%) had N2 regional LN, 47 cases (85.45%) had no distant metastasis, 27 cases (49.09%) had stage III, 36 cases (65.45%) had positive Estrogen receptor 29 cases (52.72%) had positive progesterone receptor, 27 cases (49.09%) had positive HER2\ neu, left side of breast is the most side affected in studied patients (52.72%), 28 cases (50.9%) were postmenopausal, and most of patients had Luminal B (41.81%).

Table (4): The relative gene expression of Annexin A3 in different groups.

	Control N= 20	**Benign N= 15**	**Carcinoma N= 55**	**P-value**
Annexin A3 (ng/ml)				
Mean± SD	11.69±4.48	12.97±2.29	16.01±2.49	**P1: ≤0.001*** **P2: 0.430** **P3: ≤0.001*** **P4: 0.002**

According to relative gene expression of Annexin A3, table 4 shows that a highly statistically significant variance was observed among control, benign & carcinoma groups.

Table (5): Association of annexin A3 level with the examined clinic pathological characteristics in BC

Variables	**Number of patients**	**AnnexinA3 Mean ± SD**	**P value**
Grade			
-II	41	16.21±2.46	0.38
-III	14	15.61±3.1	

Variables	Number of patients	AnnexinA3 Mean ± SD	P value
TNM stage			
-I + II	22	14.66±2.11	0.001*
-III + VI	33	16.81±2.45	
Pathological tumor type			
IDC	52	15.97±2.49	0.18
Ductal carcinoma insitu	3	13.99±0.54	
Distant metastasis			
M0	47	15.57±2.30	0.002*
M1	8	18.59±2.89	
Menopausal status			
-Premenopausal	27	15.01±2.12	0.06
-Postmenopausal	28	16.12±2.22	
Estrogen receptor			
-Positive	36	16.10±2.33	0.57
-Negative	19	15.67±3.12	
Progesterone receptor			
-Positive	29	16.75±2.4	0.009*
-Negative	26	14.95±2.48	
HER_2\ neu			
-Positive	27	16.31±1.70	0.19
-Negative	28	15.40±3.11	
Molecular type			
-Luminal A	14	15.67±3.13	0.44
-Luminal B	15	16.45±1.57	
-Triple Negative	23	15.0±3.25	
-HER2 enriched	3	16.49±1.01	

Table 5 shows that AnnexinA3 showed significant relation with TNM stage, distant metastasis, progesterone receptor.

Table (6): Sensitivity, specificity & area under ROC curve of annexin A3 within BC cases.

	Sensitivity	Specificity	AUC	sig	Accuracy
AnnexinA3	84.13%	66.53%	0.75	.000	76.7

AUC: area under ROC curve

Table 6 shows that AnnexinA3 had sensitivity of 84.13% & specificity of 66.53% in BC patients' diagnosis.

Discussion

The recent research showed that a statistically insignificant variance was observed regarding age at menarche (years) and age at marriage (years), and a statistically significant variance was detected regarding family history of BC, while a highly statistically significant difference was observed regarding age at diagnosis, number of deliveries, and breast-feeding duration in years between the control, Benign, and carcinoma groups.

Posso et al. [8] performed a nested case-control study. Females with breast cancer (BC) & previous benign breast diseases (BBDs) (86 cases) have been matched with females with previous benign breast diseases that have been free from BC (172 controls). The authors reported that insignificance was observed among the examined groups with regard to age at menarche (years) and age at marriage (years).

Zhou et al. [9] who aimed to identify an association among the expression of ANXA3 &the prognosis of BC, Among the

309 cases with primary breast cancer who participated in the research, the median age was 47 years, & the age range was 22–76 years.

Dorjgochoo et al. [10] demonstrated that a statistically significant variance was detected with regard to family history of BC, while a highly statistically significant variance was detected with regard to age at diagnosis, number of deliveries, and breast-feeding duration in years between Control and Benign.

Our findings showed that 3 cases (5.45%) had ductal carcinoma insitu and others had IDC in the carcinoma group, while 12 cases (80%) had fibroadenoma and others had fibrocystic diseases in the Benign group. According to the clinicopathological characteristics of the breast cancer patients, table 5 shows that most patients had grade II (74.54%), most patients had T3 tumors (41.81%), 22 cases (40%) had N2 regional LN, 47 cases (85.45%) had no distant metastasis, 27 cases (49.09%) had stage III, and 36 cases (65.45%) had a positive estrogen receptor. 29 cases (52.72%) had positive progesterone receptor, 27 cases (49.09%) had positive HER2\ neu, the left side of the breast is the most affected in the studied patients (52.72%), 28 cases (50.9%) were postmenopausal, and most of the patients had Luminal B (41.81%).

Our results were supported by Dorjgochoo et al. [10], who demonstrated that the most affected side of the breast in the studied patients was the left side. Also, Figueroa et al. [11] & Anastasiadi et al. [12] reported similar results**.**

The current study showed that according to relative gene expression of annexin A3, table 4 shows that a highly statistically significant variance was observed among the control, benign, & carcinoma groups. AnnexinA3 showed a significant relation with TNM stage, distant metastasis, and the and the progesterone receptor. AnnexinA3 had a sensitivity of 84.13% and a specificity of 66.53% in the BC patients' diagnosis.

Our results are supported by Du et al. [13] who reported that a negative association was detected among ANXA3 expression & BC prognosis. They demonstrated that ANXA3 is significantly upregulated in breast tumor tissues obtained from clinical biopsies. The IκBα-mediated mesenchymal-epithelial transition & the transition of various stages of BCSCs were the causes of the fact that ANXA3 knockdown suppressed BC cell infiltration and enhanced proliferation in vitro & in vivo. Furthermore, they demonstrated that the uptake of doxorubicin was facilitated by ANXA3 knockdown, and the inhibition of ANXA3 in conjunction with doxorubicin might effectively prevent tumor growth and metastasis.

Our outcomes were supported by Ozturk [7]**,** who reported that annexins, proteins found in various cell types, play roles in various processes. They are associated with various diseases, with annexin A3 (ANXA3) being linked to malignant tumors like breast cancer. ANXA3 expression in breast cancer cells is poor prognostic, increases invasion ability, and may be a potential therapeutic target.

Also, Kim et al. [14] demonstrated that the expression of ANXA3 was closely related to tumor size, with a greater level of ANXA3 expression correlated with a decreased disease-free survival in BC cases.

Furthermore, Zhou et al. [15] BC prognosis is correlated with elevated expression of ANXA3. The expression profile is dependent upon the subtype of BC; patients with triple-negative BC exhibited the highest expression levels. The research, therefore, links ANXA3 to prognosis and suggests that it may be involved in the development, metastasis, and occurrence of BC.

As evidenced by immunohistochemistry and western blot outcomes, ANXA3 has been overexpressed in colorectal cancer (CRC) tissues in comparison to surrounding normal tissues [16].

Conclusion

We concluded that Annexin A3 is a reliable diagnostic marker for breast cancer. Further prospective studies with larger scales are needed to confirm our results.

Conflict of interests

There is no conflict of interest. The manuscript has been read and approved by all the authors, that the requirements for authorship as stated earlier in this document have been met, and that each author believes that the manuscript represents honest work.

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