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RESEARCH ARTICLE

Clinical Study of Phospholipase D1 and Choline Kinase Alpha Enzymes in Women with Breast Cancer in Thi-Qar Governorate-Iraq

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Abstract

Objective: Breast cancer is the most common cancer among women, comprising 18% of all female cancers, and worldwide, breast cancer is the fifth most common cause of cancer mortality. The study was designed to determine and compare the levels of phospholipase D1 (PLD1) and choline kinase alpha (CHKAα) enzymes in Breast cancer patients and apparently healthy individuals. Material and Methods: Blood phospholipase D1 (PLD1) and choline kinase alpha (CHKAα) enzymes levels were determined in 85 breast cancer patients and 55 apparently healthy subjects. Histopathological and immunohistochemical analysis done for (50) cases (out of those 85 breast cancer patients), tissue samples are collected from patients with breast cancer who undergoing surgical resection (mastectomy) and normal breast tissues: (control group, which include normal breast tissue from patients with benign lesions, and normal breast tissue from the same women with breast cancer). Results: The levels of serum phospholipase D1 (PLD1) and choline kinase alpha (CHKAα) enzymes were showing significant increase in breast cancer patients as compared to control group ($P \leq 0.05$). We found that 82% of breast cancer biopsies were phospholipase D1 positive. There was no expression of phospholipase D1 in all normal breast tissues. Sixty-seven percent of breast cancer biopsies were choline kinase alpha positive. There was no expression of choline kinase alpha in all normal breast tissues. Conclusion: In Breast cancer patients, we found that there is an increase in (PLD1) and (CHKAα) enzymes level both in serum and malignant tissue, and this suggests that the overexpression of PLD1 and CHKAα may play an important role in the human breast tumorigenesis and they are useful markers for breast cancer.

Keywords: *Breast cancer, Phospholipids, Phospholipase D1 (**PLD1**), Choline kinase alpha (**CHKAα**).*

Introduction

Breast cancer is the most common cancer among women, comprising 18% of all female cancers, and worldwide, breast cancer is the fifth most common cause of cancer mortality [1]. According to a recent report published by the American Cancer Society, breast cancer is the most common type of cancer in women in the USA.

In 2017 alone, studies indicate that approximately 252,000 new cases of invasive breast cancer and 63,000 cases of in situ breast cancer are expected to be diagnosed, with 40,000 breast cancer-related deaths expected to occur [2]. Iraqi National Cancer Research Center (INCRC) considered breast cancer as common type of Iraqi female cancer, account for approximately (1:3) of the registered female cancers in Iraq (INCRC, 2016).Phospholipids, which shape the bi-layer structures of all cellular membranes, are an essential component of all cells. The phospholipid content was appeared to increase with cell transformation and tumor progression [3].PLD is an enzyme that belongs to the phospholipase super family [4, 6].

Classical PLD enzymes hydrolyze the abundant membrane lipid phosphatidylcholine to yield the second messenger phosphatidic acid (PA) and choline [4]. Most vertebrates have two classic isoforms, and PLD2 encode an identical series of protein regions, including Pleckstrin homology (PH) and Phox (PX) domains and a

phosphatidylinositol 4,5-bisphospate-interacting motif that direct relationship with specific subcellular membranes during signaling events, in addition to the pair of split catalytic domains [7, 8]. Potential roles for PLD in general or for PLD1 particularly have been reported in various physiological settings including ones relevant to cancer for example, survival signaling [9], control of cell polarity [10].Ras activation [11], and cell migration [12].

Moreover, a PLD1 single nucleotide polymorphism (SNP) relates with the risk of non-small cell lung cancer and increased PLD1 expression and/or PLD activity have been accounted in multiple types of cancer although the mechanisms underlying this increase and the specific advantage this gives to the tumor cells are not known [13]. Choline kinase (ChoK) is an enzyme encoded by two genes, CHKA and CHKB, which catalyzes the phosphorylation of free choline in the cytoplasm using ATP to generate phosphocholine. Three isoforms of ChoK are known in mammalian cells: ChoKα-1, ChoKα-2, and ChoKβ, and the active shape of the enzyme consist of a hetero- or homo-dimer [14].

ChoK is the first enzyme in the Kennedy pathway, which is responsible for the synthesis of phosphatidylcholine and phosphatidylethanolamine. ChoKα expression and activity have been found up regulated in several types of cancer, including lung, prostate, colorectal, breast, ovarian, and bladder becoming an attractive potential therapeutic target in oncology [15, 16].ChoK is regulated by several growth factors and hormones, can be induced via carcinogens [14], and contributes to malignant transformation and progression. In this study we measure the serum level of phospholipase D1and choline kinase alpha and also we detect these enzymes in malignant tissue by immunohistochemistry in women with breast cancer.

Material and Methods

This study designs as prospective study, all samples are taken from patients who attended the oncology unit in Al-Habboby Hospital and specialist clinics. Including (85) blood samples from patients with breast cancer, (55) blood samples are collected from healthy women as a control group.

About (5mL) of blood samples of breast cancer patients and controls were taken and allowed to clot at room temperature in empty disposable tubes centrifuge to separate it in the centrifuge at 3000 rotors per minute (rpm) for10 min, the serum samples were separated and stored (-20ºC) until analyzed for phospholipase D1 and choline kinase alpha enzymes. Serum PLD1 was estimated by enzyme linked immunoassay method by ELISA Reader, USA using kit supplied by Elab science, USA.Serum CHKAα was estimated by enzyme linked immunoassay method by ELISA Reader, USA using kit supplied by Al- Shkaireate, Sweilleh Amman, Jordon.

Histopathological and immunohistochemical analysis done for (50) cases (out of those 85 breast cancer patients), tissue samples are collected from patients with breast cancer who undergoing surgical resection (mastectomy) and normal breast tissues: (control group, which include normal breast tissue from patients with benign lesions, and normal breast tissue from the same women with breast cancer). Both phospholipase D1and choline kinase alpha was estimated immunohistochemically using kits labeled in Table (1). The results were expressed as mean ± standard deviations (mean ± SD). One-way ANOVA-test was used to compare parameters in different studied groups. P-values ($P \leq 0.05$) were considered statistically significant.

Table 1: List of Materials (kits) used in the study

No.	Item name	Manufacturer company	Quantity	Catalogue No.	Host	Target	Clonality
1	**PLD1 antibody**	Biorbyt (UK)	50 µL	**Orb96001**	Rabbit	Human	Polyclonal
2	**choline kinase alpha**	Biorbyt (UK)	50 µL	**Orb98213**	Rabbit	Human	Polyclonal
3	**Super Sensitive IHC Detection System Kit (Mouse/Rabbit)**	**Biorbyt (UK)**	**1kits**	**orb219874**	-	**Rabbit and mouse**	-

Results

Histological type of Breast Cancer

All breast cancer tissues (50 cases) available for histopathology were invasive ductal carcinoma.

Clinical and Characteristic Features of the Studies Groups

There are 140 subjects included in the present study, with difference in clinical characteristic breast cancer patients group (eighty-five women) were compared with group of apparently healthy control (fifty-five women) without significant difference in Age, Table (2).

Table 2: Characteristic data for studied groups

Groups	No.	Age year mean± SD	BMI(Kg/m2) mean± SD
control	55	44.67± 8.22	**23.68±2.32**
patients	85	47.63± 9.94	**31.62± 2.42**

Staging of Breast Cancer

Out of 50 cases, we found that 2 cases (4%) were stage I, 16 cases (32%) were stage II, 28 cases (56%) were stage III and 4 cases (8%) were stage IV. There was no significant difference among different stages ($P \leq 0.05$), Table (3).

Table 3: Distribution of patients according to stage

Stage	Number of patients	%
Stage I	2	4
Stage II	16	32
Stage III	28	56
Stage IV	4	8
Total	50	100

($P \leq 0.05$)

Grading of Breast Cancer

Out of 50 cases, we found that 4 cases (8%) were grade I, 21 cases (42%) were grade II, and 25 cases (50%) were grade III. There was no significant difference among different grades ($P \leq 0.05$), Table (4).

Table 4: Distribution of patients according to grade

Grade	Number of patients	%	
Grad I	4	8	
Grade II	21	42	
Grade III	25	50	
Total	50	100	

($P \leq 0.05$)

Serum Phospholipase D1 (PLD1) and Choline Kinase Alpha (CHKAα) and Breast Cancer

In this study we determined the relation between the serum phospholipase D1 (**PLD1**) and choline kinase alpha (**CHKAα**) levels and breast cancer. Phospholipase D1 (**PLD1**) and choline kinase alpha (**CHKAα**) serum level showed significant increase in breast cancer patients as compared to control group, Table (5&6) Figure (1&2).

Table 5: Serum Phospholipase D1 (PLD1) activity of (control) and (breast cancer) groups

Group	n	PLD1activity (ng/mL) mean± SD
control	55	75.24±17.06[b]
Breast cancer	85	283.68±58.79[a]
LSD		11.66

* Each value represents mean ± SD values with non-identical superscript (a, b or c …etc.) were considered significantly differences ($P \leq 0.05$)

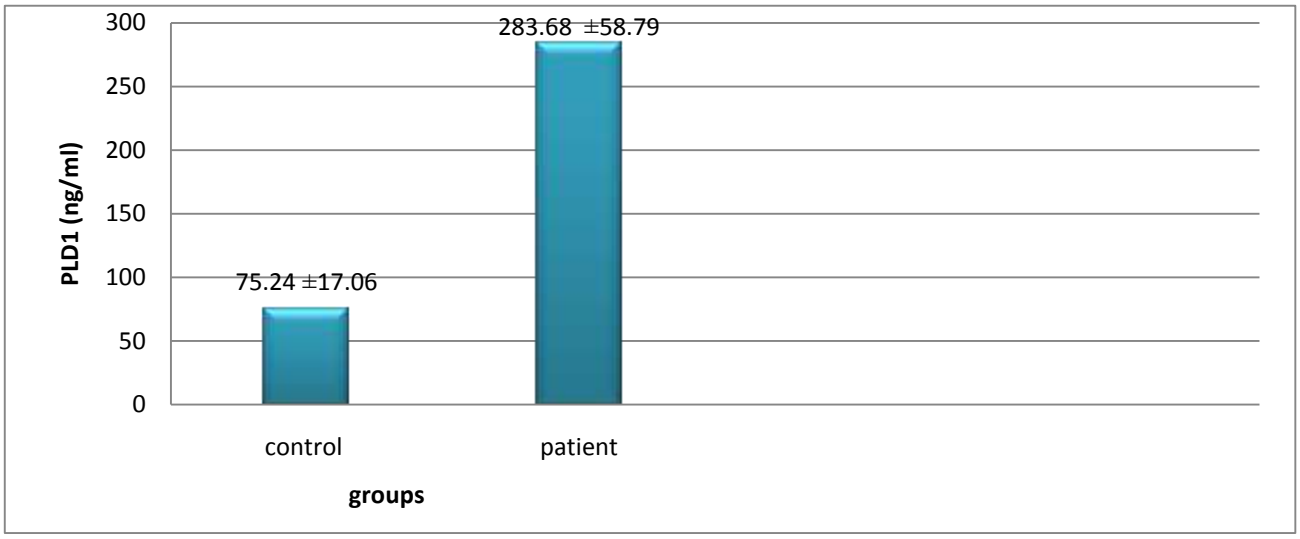

Figure 1: Serum PLD1 levels of control and breast cancer women groups

Table 6: Serum Choline kinase alpha (CHKAα) activity of (control) and (breast cancer) groups

Group	n	CHKAα activity (ng/ mL) mean ± SD
control	55	8.51±2.20[b]
Breast cancer	85	27.68±5.28[a]
LSD		1.10

- Legend as in table (5)

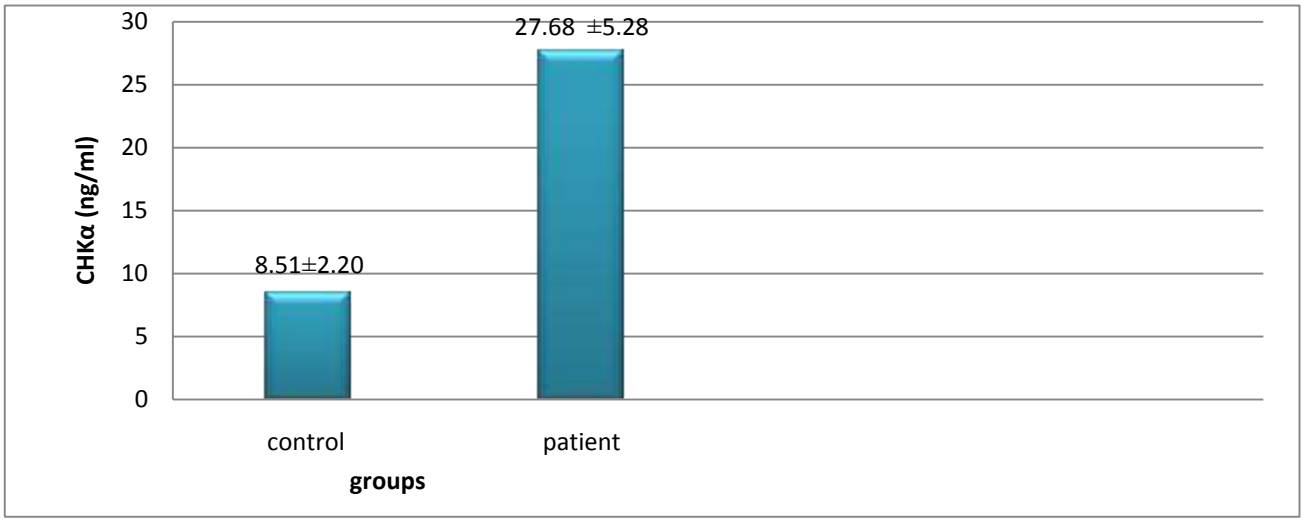

Figure 2: Serum CHKAα levels of control and breast cancer women groups

Immunohistochemistry Staining of Phospholipase D1and Choline Kinase alpha

We found that 41 cases (82%) were phospholipase D1 positive results Figure (5, 6&7), and 9 cases (18%) were negative, Figure (3). There was no expression of phospholipase D1 in all normal breast tissues (control group, which include normal breast tissue from patients with benign lesions, and normal breast tissue from the same women with breast cancer). There were 38 cases (67%) were choline kinase alpha positive results, figure (6&7), and 12 cases (24%) were negative. There was no expression of choline kinase alpha in all normal breast tissues (control group).

Immunohistochemical Staining of Phospholipase D1 and Choline Kinase Alpha according to different Grades

In this study we found that 25 cases which are positively stained with phospholipase D1 were grade III and the remaining 16 cases were grade II breast cancer. All four grade I cases were negative immunohistochemical staining for phospholipase D1. Twenty-two cases which are positively stained with choline kinase alpha were grade III and the remaining 14 cases were grade II breast cancer. All four grade I cases were negative immunohistochemical staining for choline kinase alpha.

Staining Scoring

The scoring of expression in the current study classified into (-, +, ++ and +++) according to the number of cells positively stained with immunohistochemical markers. Brown cytoplasmic staining was considered positive reaction and compared with the cytoplasmic staining of the positive control slide that used in this study which is normal splenic tissue, Figure (3). Classification of the cases of carcinoma, and control groups into different grades of intensity (-, +, ++ and +++) showed no significant difference ($P \leq 0.05$) for both phospholipase D1 and choline kinase alpha. In the 41 cases which were positive stain for phospholipase D1; staining with score +++ was seen in 14 (28%) cases, score ++ was seen in 18 cases (36%), and 9 (18%) cases were score +, and 9 (18%) cases of breast cancer were negative, (Table 7), Figures (5, 6, 7). The scoring of expression of choline kinase alpha in the 36 positive cases; score +++ was seen in 10 (20%) cases, score ++ was seen in 17 cases (34%), and 11 (22%) cases were score +, and 12 (24%) cases of breast cancer were negative, (Table 7), Figures (6, 7).

Table 7: Frequency distribution of immunohistochemical expression of Phospholipase D1andcholine kinase alpha in breast cancer, and control groups

				Study groups		
				Breast cancer N (%)	**Control 1 N (%)**	**Control 2 N (%)**
IHC score	-		***Phospholipase D1***	9(18%)	50 (100%)	35(70%)
			choline kinase alpha	12(24%)	50(100%)	28(56%)
	+		***Phospholipase D1***	9 (18%)	0 (0%)	0 (0%)
			Choline kinase alpha	11(22%)	0 (0%)	0 (0%)
	++		***Phospholipase D1***	18 (36%)	0 (0%)	0 (0%)
			choline kinase alpha	17(34%)	0 (0%)	0 (0%)
	+++		***Phospholipase D1***	14(28%)	0 (0%)	0 (0%)
			choline kinase alpha	10(20%)		
Total				50(100%)	50 (100%)	50 (100%)
P value				($P \leq 0.05$)		

****Note: Control 1**(normal breast tissue from the same women with breast cancer)
Control 2 (normal breast tissue from patients with benign lesions)

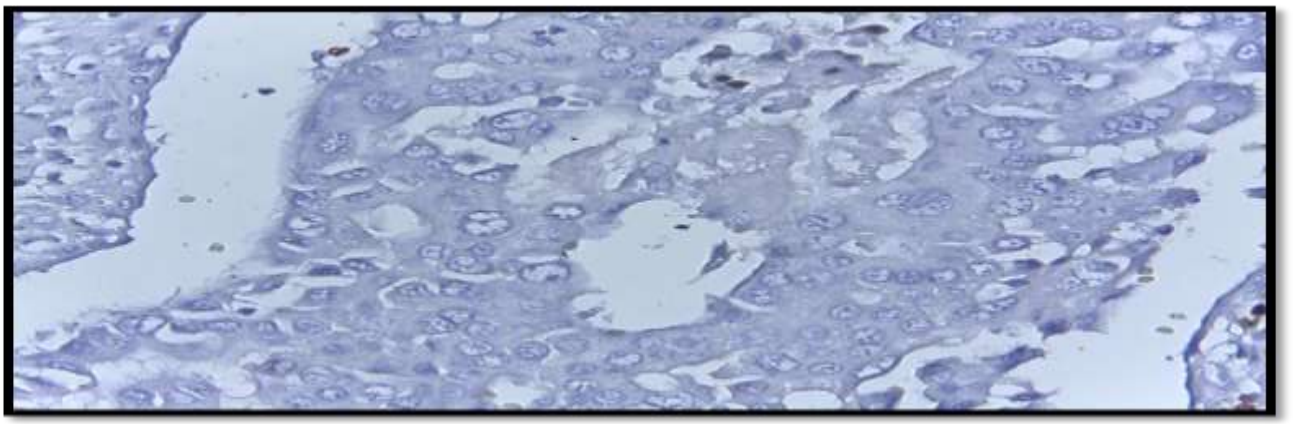

Figure 3: Invasive ductal carcinoma, showing negative expression of phospholipase D1, negative control (40X)

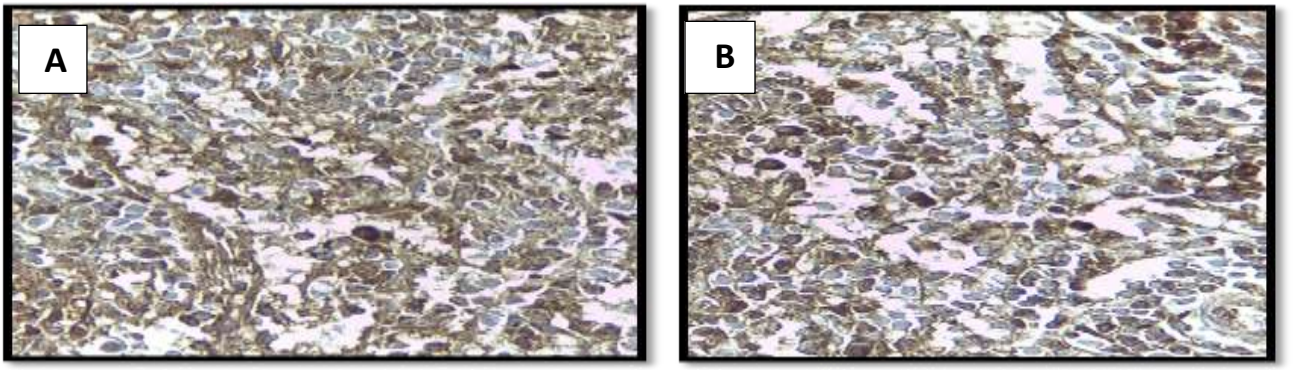

Figure 4: A; positive control, splenic tissue showing positive expression of phospholipase D1, B: positive control, splenic tissue showing positive expression of choline kinase alpha (10X)

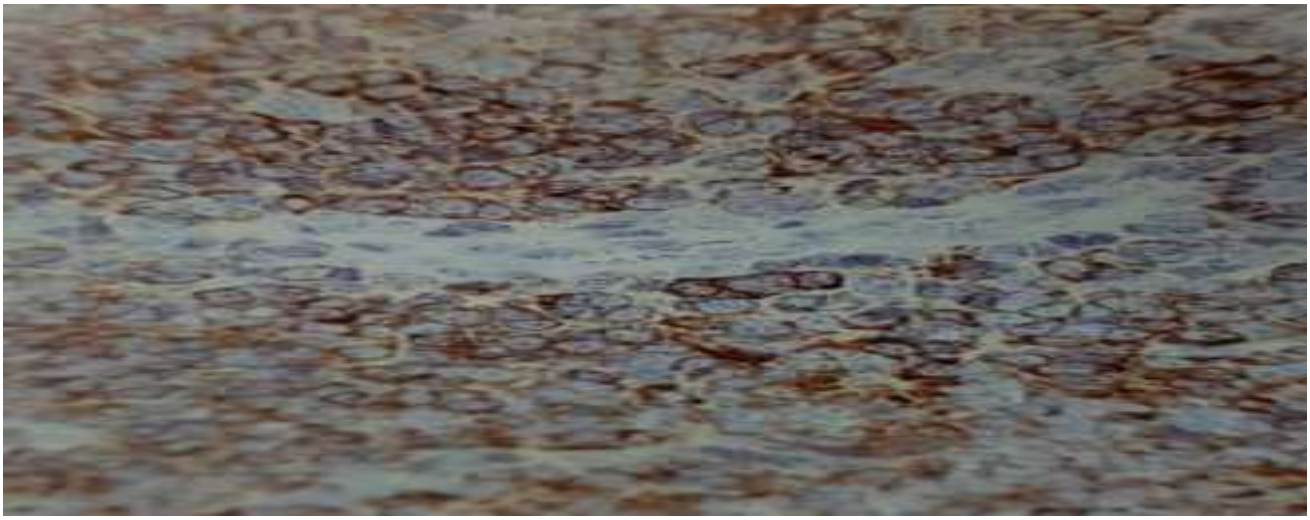

Figure 5: Invasive ductal carcinoma, showing positive expression of phospholipase D1 (+++) (40X)

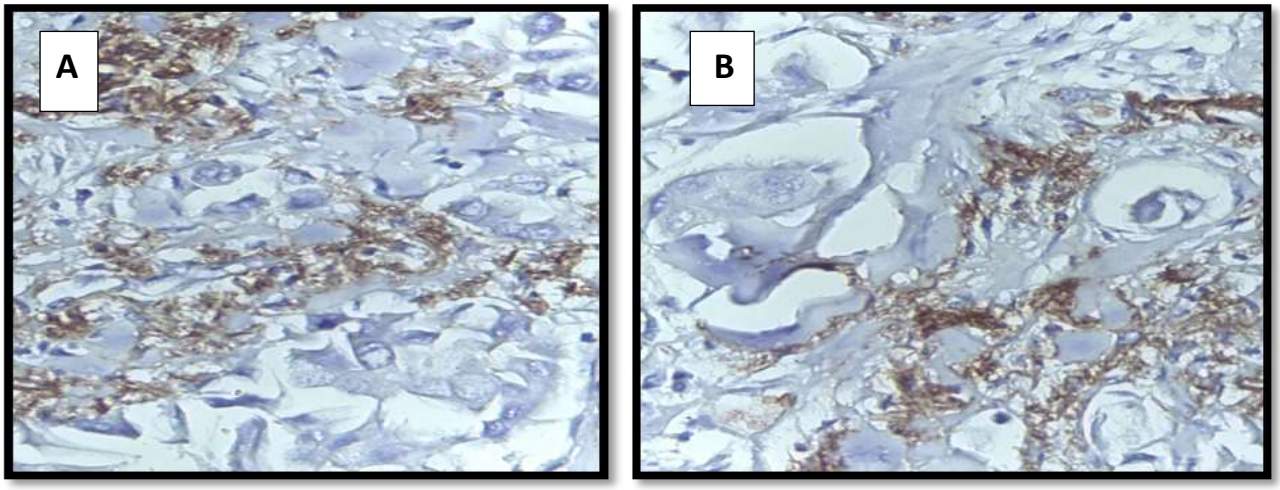

Figure 6: Invasive ductal carcinoma, showing moderate positive expression of: A phospholipase D1 (++), B choline kinase alpha (++) (40X)

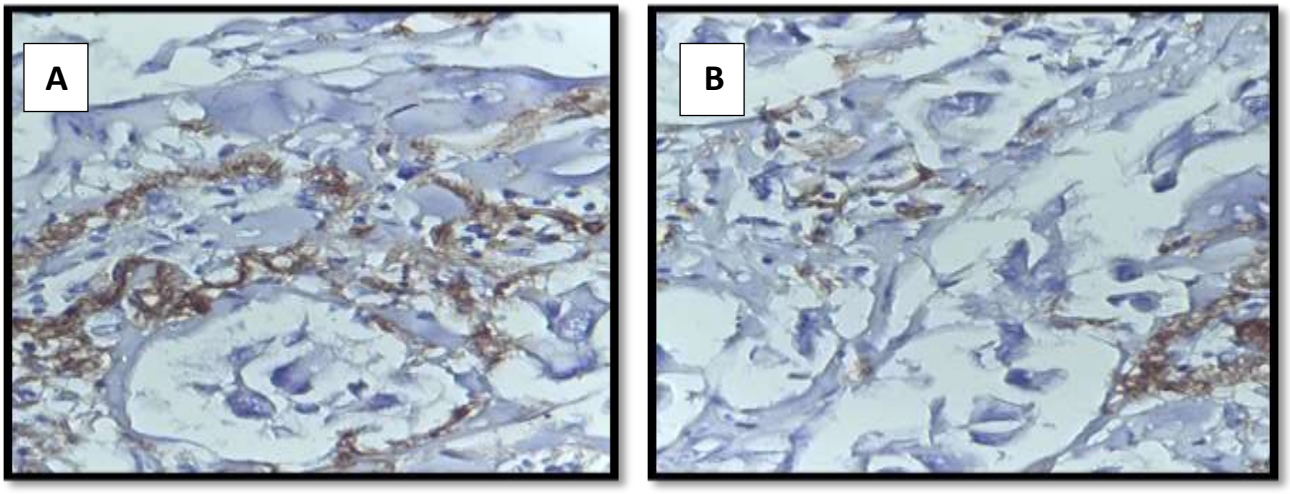

Figure 7: Invasive ductal carcinoma, showing weak positive expression of: A phospholipase D1 (+), B choline kinase alpha (+) (40X)

Discussion

Our results regarding age, staging and grading agree with many Iraqi studies [17, 19]. So most cases in present study are advance stage and grade. These observations obviously reflect the poor health education of the general population and their ignorance regarding the significance of clinical breast examination, breast self-examination and early medical consultation. Clinical studies have reported on correlation of PLD expression levels with tumor size; an association with survival of patients with cancers was also accounted. PLD activity is increased significantly in cancer tissues and cells, showing that it may play a critical role such as signal transduction, cell proliferation,

and anti-apoptotic processes [20]. The phospholipases generate numerous lipid products which control much of cellular signaling. Although the importance of these enzymes in eicosanoid metabolism signal translation is not addressed, much remains to be studied regarding the regulation of these enzymes and their pathophysiological roles.

Recent large-scale metabolomics and lipidomic profiling studies of human tumors and patient blood support the hypothesis that the metabolic phenotype of a tumor reflects its genomic pattern and signaling pathway status [21, 23].The changes in relative gene expression of enzymes involved in choline handling have been studied in a various of tumor types including breast and ovarian cancers, and these studies are consistent in reporting an increase in the expression and activity of CHKA in cancer cells compared to normal epithelial cells [24].This is leading to new opportunities in accuracy medicine: the utilization of tumor metabolic fingerprinting as a diagnostic tool to enhance choice of patients for targeted therapies, and the identification of tumor-specific metabolic vulnerabilities for novel therapeutic approaches in cancer.

Conclusion

We can conclude in this study that there is a significant increase in serum phospholipase D1 (PLD1) and choline kinase alpha (CHKAα) enzymes in patients with breast cancer, both with overexpression of PLD1 and CHKAα in malignant tissue of patients with breast cancer. This conclusion suggests the role of those two enzymes in the human breast tumorigenesis and their importance as markers for breast cancer and a therapeutic target in oncology.

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