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LOSS OF IMMUNOHISTOCHEMICAL EXPRESSION OF PTEN AS A PREDICTIVE BIOMARKER IN BREAST CARCINOMA

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Abstract - Background: Breast cancer is the most common malignancy with a high mortality in females worldwide. Tumor suppressor genes had significant role to maintain genome integrity and the cell cycle. In particular PTEN is a candidate tumor suppressor gene, It has a negative regulator of PI3K/AKT signaling pathway which has a major role in carcinogenesis and dysregulation of it occurs repeatedly in breast cancer. Aim: this article aimed to appraise the associations between PTEN expression in patient had breast cancer with clinic pathological parameters including: age, histological types, and status of estrogen receptor, progesterone receptor, and HER-2/nue receptor, to check the impact of its expression on clinical outcome. Materials and methods: in this case-control study, formalin fixed paraffin embedded tissue from sixty patients with breast carcinoma and twenty patients without cancer (as control groups). Labeled Streptavidin -Biotin (LSAB$^+$) method used to detect PTEN protein expression, HER2/neu, ER and PR receptors by immunohistochemical assay, and then we correlate PTEN expression with each biomarkers and clinic pathological characteristics. Results: 29/60 (45.3%) of cases decreased PTEN expression while its expression retained in 31/60 (51%) of cases. Loss of expression significantly associated: with lymph node metastasis (p-value=0.0008), high grade (p< 0.05) , high stage (p-value=0.0001) and with triple negative breast cancer (p-value=0.03) . However, loss of PTEN protein expression did not correlate with age, histological types, estrogen, progesterone and HER-2 receptors status. Conclusion: PTEN loss can predict aggressive behavior and worse outcome in patients had breast cancer.

Keywords: PTEN protein expression, immunohistochemistry, breast cancer.

INTRODUCTION

Breast cancer is the most common malignancy among women in both developing and developed countries, represent one fifth of new cancer cases in female [1]. PTEN (Phosphatase and Tensing Homolog deleted on chromosome 10) was candidate tumor suppressor gene, is localized on chromosome 10q23 and shares extensive homology with cytoskeleton proteins auxilin and tensing .It had a negative regulator of PI3K/AKT signaling pathway that influence cell metabolism, proliferation, apoptosis, survival [2]. Overall, the PI3K/ PTEN pathway has a major role in carcinogenesis. Dysregulation of it occurs repeatedly in breast cancer. **The Phosphoinositide 3-kinase/ Akt Pathway:** The lipid phosphatase function of PTEN acts as a negative regulator of the AKT/pathway. PTEN dephosphorelates (PIP3) at the D3 position generating (PIP2), thus decreases the cellular (PIP3) levels (Figure-1) [3-5].

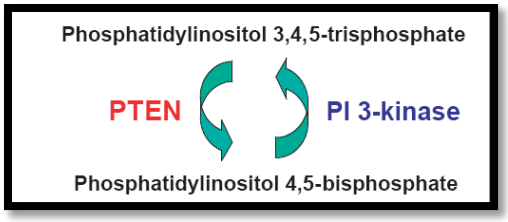


Figure (1): PTEN a lipid Phosphatase. PI3K lipid kinase catalyzes the transfer of phosphate group to PIP2, thus generating PIP3. PTEN removes the phosphate group, and regenerates PIP2

Furthermore, PTEN loss may affect tumor genesis and the incidence of tumor growth in vivo by increasing tumor cell invasiveness. Recently, HER2/ErbB2 activation of PI3K-dependent signaling potentially increases invasiveness of mammary epithelial cell in vitro [6]. In addition, PTEN dephosphorelates focal adhesion kinase and inhibits integrins-mediated cell migration and cell spreading [7]; thus, decreased PTEN expression may support a metastatic behavior. In breast cancer, there is emerging evidence suggesting that loss of function of PTEN not only plays role in tumor genesis, but also it may be a key role in resistant to targeted therapy [8].

MATERIAL AND METHOD

(a) Study group: 60 female patients had breast carcinoma (confirmed with histopathological examination with H&E stain) had been involved in this work, their ages ranging from 24 to 70 years (mean age about 44.9), all samples had been taken from modified radical mastectomy. All samples had been taken from histopathological laboratory in AL-Saddar Teaching hospital in Najaf and from some private laboratories in the similar site during the period December 2018- December 2019.

(b) Control group: 20 samples of normal breast tissue had been taken from patients with identical age groups, for reasons other than breast cancer, and made them control group.

The original diagnosis for each case was re-evaluated to confirm the presence of tumor using Hematoxylin-Eosin stain and to re-evaluate the grade of the tumor. All cases were evaluated for ER, PR, HER-2, and for PTEN by Immunohistochemistry

MATERIALS AND EQUIPMENTS

Primary Antibody

A- Monoclonal Mouse Anti-Human PTEN Clone 6H2.1: This antibody is intended to identify PTEN expression under light microscopy in normal and neoplastic tissue cells using immunohistochemical (IHC) test.

B. HER2/neu: Polyclonal Rabbit Anti- Human c-erbB-2 Oncoproteinhave, 0.2 ml, Code No. A0485, A/S, Produktionsvej 42, LOT 00029863, Dako Denmark DK-2600 Glostrup, Denmark was used as primary antibody for the detection of HER -2 /neu protein.

C. Estrogen: Monoclonal mouse anti-human estrogen receptor α, 0.2ml/1ml, Code No. M7047, Dako cytomation Denmark A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark was used as primary antibody for the detection of estrogen receptor.

D. Progesterone: Monoclonal mouse antihuman progesterone receptor, 0.2ml/1ml, Code No. M3569, Dako cytomation Denmark was used as primary antibody for the detection of progesterone receptor.

Protocol of immunohistochemical staining

The immunostaining method used in this work for staining of PTEN, HER2/neu, ER, and PR was En Vision immunohistochemical technique which involved the followings [9]:

Tissues: paraffin-embedded blocks had been made from 10% formalin fixed human tissues the sectioning was done by microtome to made thin slices (usually with 4 µm thickness), mounted on Silanized slides.

Primary antibody: Antibodies were diluted in Dako Antibody Diluents.

Control: The negative control was pretreated and keeps within Antibody Diluents step in this protocol.

Deparffinzation: it had been done previously by immersion in the followings: (The sections had been dried at 60 C about 1 hour).

1. Xylene for 5 minutes.(2 times)

2. 99 % ethanol for 5 minutes. (3times)

3. 95 % ethanol for 5 minutes.

4. 70 % ethanol for 5 minutes.

5. Distilled Water.

Pretreatments: [Heat induced epitopes retrieval in MWO (microwave oven)]

a. Heating fluid about 250ml, 10/1 mM Target Retrieval solution, PH9, had been teemed into a plastic can. Slides had been placed into a plastic slide holder then transported to the plastic cans, replenish the slide holder with slides, as a result the slides number was the same every run. The cover is put on and the plastic can had been entered the microwave. Every run similar numbers of cans had been heated cans without slides should include 250 ml distilled water.

b. Put microwave at highest degree untill the fluid seethes.

c. Put microwave at midst degree (nearly 350W) and heat about fifteen minutes.

d. Remove cans from microwave then allow the slides rest in the hot fluid for 20 minutes.

e. Put cans under gentle rinse water about five minutes.

f. Proceed as the subsequent immunoprotocol

- Irrigate within (TBS) Tris Buffered Saline, about five minutes
- Border on the tissue with Pap Pen .Erase buffer 1/2 cm below and above the tissue and lined it with the pap pen.
- Irrigate within (TBS) about five minutes
- Incubate with Peroxides Blocking-Reagent about ten minutes
- Irrigate within (TBS) about five minutes
- Incubate in Primary Antibody Incubate* about thirty minutes
- Irrigate within (TBS) about 2×5 minutes
- Incubate with Biotinylated Link Antibody (K0679) * about fifteen minutes
- Irrigate within (TBS) about 2×5minutes
- Incubate with Streptavidin/Peroxidase about fifteen minutes
- Irrigate within (TBS) about 2×5 minutes.
- Incubate with DAB+ about ten minutes
- Irrigate within (TBS) about two minutes
- Irrigate within distilled water about two minutes
- Count Stain in Mayer's Hematoxylin about two minutes
- Irrigate within rinsing water about five minutes

- Mount with Far amount or dehydrate and cover slip the slides. (The slides must not dry out during the whole procedure).

Interpretation of staining
Immunohistochemical staining for ER, PR, was assessed with Allred scoring system described in most recent American Society of Clinical Oncology/College of American Pathologists guidelines. Briefly, nuclear staining of the invasive tumor cells was designated an intensity score:

0=no staining, 1=weak staining, 2=moderate staining, 3=strong staining

Proportion score
0=no staining, 1=<1%, 2=1-10%, 3=11-33%, 4=34-66%, 5=67-100%.

Then the intensity and proportion score summed to give total score range from 0- 8. PTEN Stained slides had been grouped as:

0= Negative, 1=Weak, 2=Moderate, 3= Strong.

Pp: (percentage of positive cells) had been explained as:

0=<5%, 1=5-25%, 2=26-50%, 3=51-75%, 4=>75%.

10 visual fields from different areas of each tumor had been used for assessment

IRS =0-3 this regard no expression (0)

IRS = 4-6 this regard low expression (1+)

IRS =7-9 this regard intermediate expression (2+)

IRS =10-12 this regard high expression (3+).

If IRS < 7 had been regarded negative, if IRS $\geq$ 7 had been regarded positive

Slides had been assessed by an expert histopathology's under light microscope.

Assessment of tumor grade and stage
Grading had been done according to the modified Bloom and Richardson criteria, while patients staged based on criteria described in the 6th edition of the AJCC guidelines [10].

Statistical analysis
Sampling of the Study: in this case-control study, population consisted of one section, which covers the patients who are suffering from breast cancer. Sixty cases of paraffin – embedded tissues were selected by Simple Random Sampling with twenty samples of paraffin – embedded tissues from patient with breast lesion but without breast carcinoma as control groups, their clinical database taken from the archives. The study includes the variables PTEN, ER, PR, HER-2, age, stage, and lymph node metastasis.

Statistical treatment: Statistical comparisons made by SPSS software statistical package (version 15) using Chi Square test, if P value < 0.05 had been accepted as statically significant and correlation regression test (R at a significant level of 0.3) .

Correlation between PTEN expression and clincopathological parameters of breast carcinoma table (1)

Table (1): Relation between immunoexpression of PTEN and clinic pathological parameters of breast carcinoma

Parameters	Total number of patients		PTEN immunoexpression				P value
			Positive		Negative		
	No.	%	No.	%	No.	%	
Type of breast tissue	20	(25)	20	(100)	0	(0)	P<0.05

Normal Malignant	60 (75)	31 (51.7)	29 (48.3)	
Age of the patient ≤ 50 year >50 year	36 (60) 24 (40)	17 (47.2) 14 (58.3)	19 (52.8) 10 (41.7)	P>0.05
Histological type Lobular carcinomas Ductal carcinomas including: Pure IDC IDC + DCIS Pure DCIS	3 (5) 57 (95) 46 (80.7) 8 (14) 3 (5.3)	2 (66.6) 29 (50.9) 25 (54.3) 2 (25) 2 (66.6)	1 (33.4) 28 (49.1) 21 (45.7) 6 (75) 1 (33.4)	P>0.05 P>0.05
Tumor grade I II III	14 (23.3%) 18 (30%) 28 (46.6%)	13 (91.7%) 7 (11.6%) 8 (13.3%)	1 (1.6%) 10(16.6%) 20 (33.3%)	P<0.05

Correlation between PTEN expression and tumor stage

From 29 (48.3%) out of 60 cases that showed loss PTEN expression: 2 (6.9%) cases in stage I, 5 cases (17.3%) in stage II, 7 cases (24.1%) in stage III, and 15 cases(51.7%)in stage IV,

And from 31(51.7%)out of 60 cases that showed PTEN expression: 14 (45.1%) cases in stage I , 9 cases (29%) in stage II, 6 cases (19.4%) in stage III ,and 2 cases(6.9%)in stage IV.

There was significant differences among these groups (P<0.05).

Table 2: The frequency and percentages of PTEN expression with respect to early and advanced stage

PTEN	Stage I	Stage II	Stage III	Stage IV	Total
NO. Negative %	2 3.3%	5 8.3%	7 11.7%	15 25%	29 48.3 %
NO. Positive %	14 23.4%	9 15%	6 10%	2 3.3%	31 51.7 %
Total NO %	16 26.7%	14 23.3%	13 21.7%	17 28.3%	60 100%

X^2=20.11, p-value: 0.0001

PTEN immunoexpression in relation to the absence or presence of lymph node metastasis

Out of 60 cases, 28 cases (46.6%) of PTEN negative breast carcinoma and 16 cases (26.7%) of PTEN positive breast carcinoma are with auxiliary lymph node metastasis.

While only one case (1.7%) of PTEN negative breast carcinoma and 15 cases (2.5%) of PTEN positive breast carcinoma are without auxiliary lymph node metastasis. There was significant difference among these groups (P<0.05), table (3).

Table (3): expression of PTEN in breast cancer in relation to the absence or presence of lymph node metastasis

	PTEN		Total
	Negative	Positive	
Breast cancer with lymph node metastasis NO. %	28 46.6%	16 26.7%	44 73.3 %
Breast cancer without lymph node metastasis NO. %	1 1.7%	15 2.5%	16 26.7 %

X^2=15.4, p-value: 0.0008

PTEN immunoexpression in the lymph nodes with metastatic breast carcinoma

In this study we have 44 cases (73.3%) out of 60 with lymph node metastasis, 35 cases (58.3%) lost PTEN expression in their metastasized lymph node [7 out of 35 (11.7%) showed positive PTEN protein expression in breast mass but lost this expression in metastasized lymph nodes(altered expression)] ,and 9 cases(15%) retained PTEN expression. Other 16 (26.7%) out of 60 cases without lymph node metastasis, figure (2).

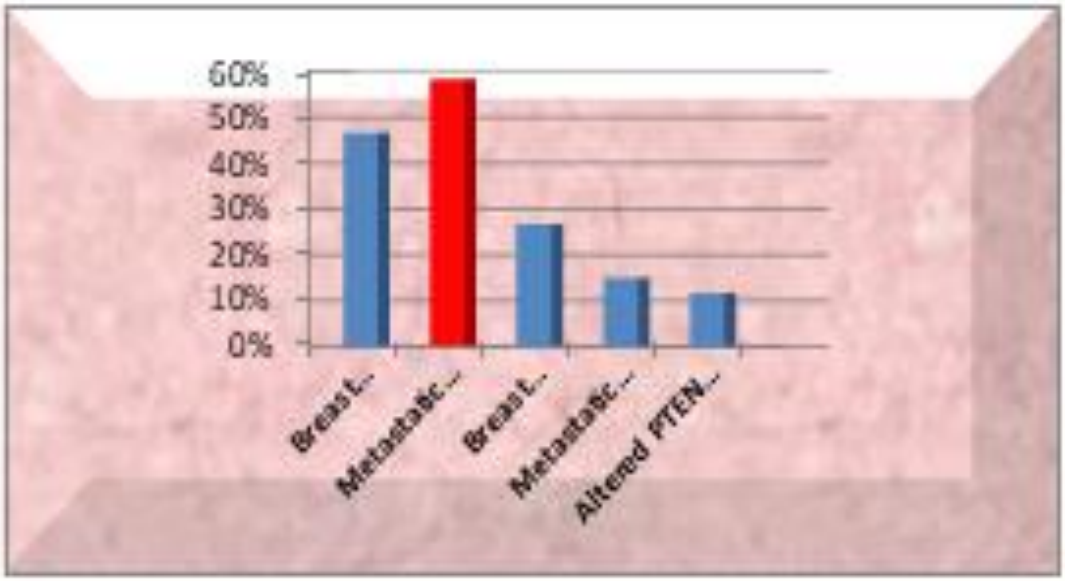


Figure (2): Altered PTEN immunoexpression in primary and metastatic breast carcinoma

Correlation between PTEN immunoexpression with other immunohistochemical markers

A-PTEN expression and Estrogen receptor status

Out of 17 ER negative cases, 9 (52.9%) lost PTEN expression and 8 (47.1%) retained PTEN expression. Of 43 ER positive tumors 20 (46.5%) were negative for PTEN expression (Figure 4.3), while 23(53.5%) cases retained PTEN expression

There was no significant difference among these groups (P>0.05) (Table -4).

Table (4): Co expression of PTEN and ER in relation to their presence or absence of expression in breast carcinoma

	PTEN		Total
	Negative	Positive	
ER Negative NO. %	 9 52.9%	 8 47.1%	 17 100%
ER Positive NO. %	 20 46.5%	 23 53.5%	 43 100%

X^2=0.02, p-value: 0.65

B- PTEN expression and Progesterone receptor status

From 45 PR positive cases, there were 22 cases (48.8%) lost PTEN expression and there were 23 cases (51.2%) retained PTEN expression. from 15 PR negative cases, there were 7 cases (46.7%) that lost *PTEN* expression and 8 (53.3%) retained PTEN expression.

There was no significant difference among these groups (P>0.05) (Table -5).

Table (5): Co expression of PTEN and PR in relation to their presence or absence of expression in breast cancer

	PTEN		Total
	Negative	Positive	
PR Negative NO. %	 7 46.7%	 8 53.3%	 15 100%
PR Positive. NO. %	 22 48.8%	 23 51.2%	 45 100%

X2=0.02, p-value: 0.88

C- PTEN expression and HER-2 receptor status

There were 35 HER-2 positive tumors, 20 (57.1%) were negative for *PTEN*, and 15(42.9%) retained their *PTEN* expression. There were 25 HER's-2 negative tumors, 9(36%) lost *PTEN* expression, while 16(64%) were *PTEN* positive

There was no significant differences among these groups (P>0.05), table (6).

Table (6): Co expression of HER2/neu and PTEN in relation to neither expression in breast carcinoma

	PTEN		Total
	Negative	Positive	
HER-2 positive NO. %	 20 57.1%	 15 42.9%	 35 100%
HER-2 negative NO. %	 9 36%	 16 64%	 25 100%

X2=2.6, p-value: 0.106

-Correlation between immunohistochemical expression of PTEN and HER-2/ER/PR status

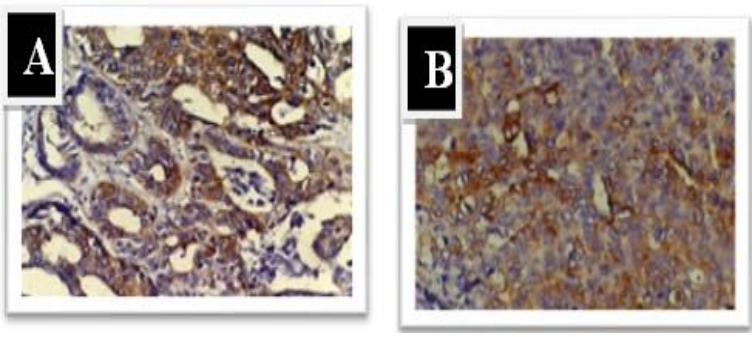
We have 13(21.6%) out of 60 cases are triple negative (HER-2 –ve, ER –ve, PR –ve) 8 of them(61.5%) lost PTEN expression ,and 5 out of 13(38.5%) show PTEN expression ,the remaining 47 cases 21(44.6%)lost PTEN expression and 26(55.4%) show PTEN expression.

There was significant differences among these groups (P<0.05), table (7).

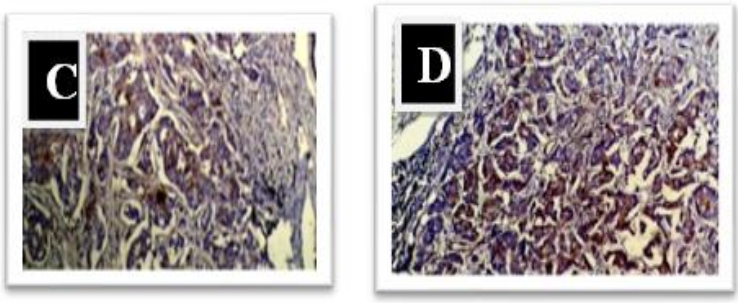
Table (7): Correlation between immunohistochemical expression of PTEN and HER-2/ER/PR status

	PTEN		Total
	Negative	Positive	
HER-2 -/ER-/PR- (Triple negative)	8 61.5%	5 38.5%	13 100%
Non-- Triple negative	21 44.6%	26 55.4%	47 100%

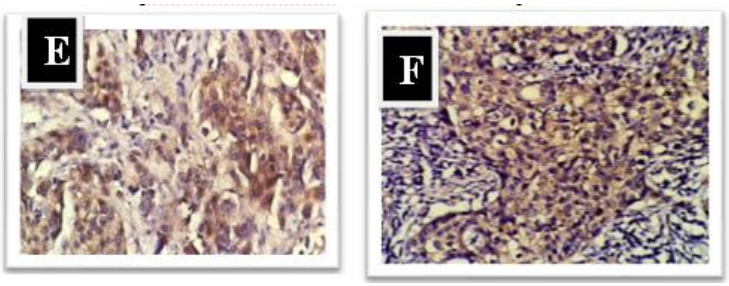
X^2=1.16, *p*-value: 0.03



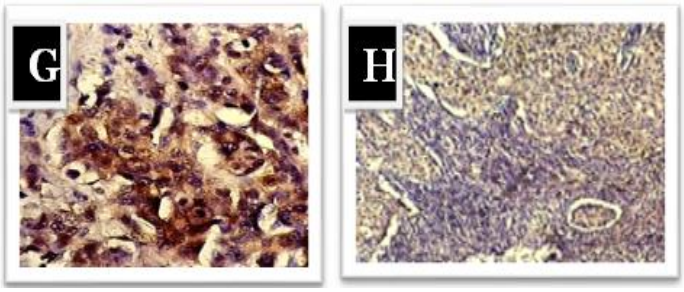
(A): Invasive ductal carcinoma (score 3+), exhibit strong cytoplasmic PTEN Staining [40X], (B): Lymph node show cytoplasmic PTEN expression of score +3[immunohistochemical stain for PTEN, 40X].



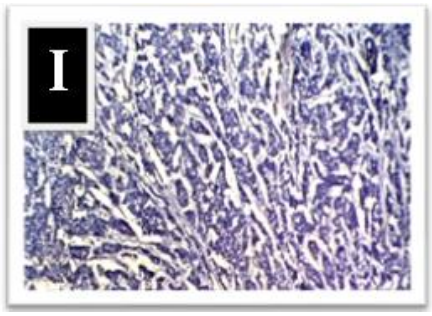
(C): Invasive ductal carcinoma, showing moderate TEN cytoplasmic staining (score 2+) [10X], (D) Lymph node show cytoplasmic PTEN expression of score+2[immunohistochemical stain for PTEN 10X].



(E)Invasive ductal carcinoma, showing weak cytoplasmic PTEN (score 1+) [40X], (F) Lymph node, cytoplasmic PTEN score +1[immunohistochemical stain for PTEN 40X].

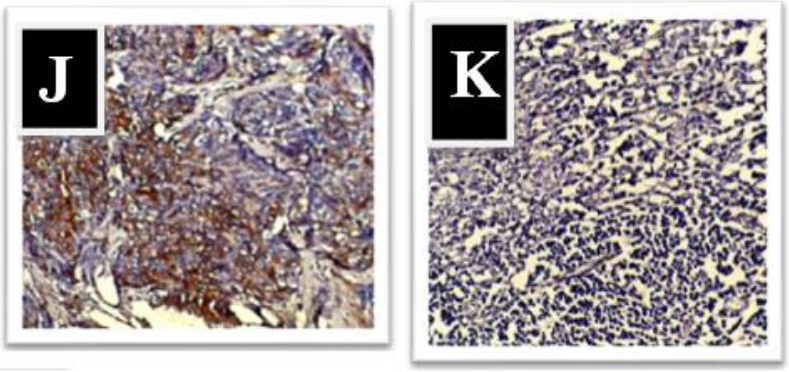


(G): Invasive ductal carcinoma, strong PTEN cytoplasmic staining (score 3+) [40X], (H): Lymph node, show cytoplasmic PTEN expression of +1[immunohistochemical stain for PTEN, 10X].

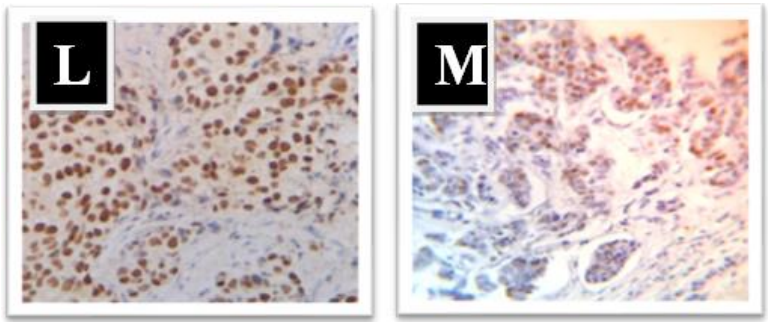


(I): Invasive ductal carcinoma, NO cytoplasmic staining (score 0), [immunohistochemical stain for PTEN, 10X].

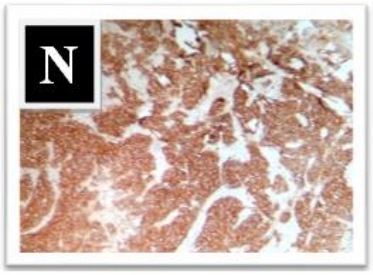
Altered PTEN expression between the mass and metastasized lymph node in the same case



(J): Invasive ductal carcinoma, PTEN strong cytoplasmic staining (score 3+) [40X], (K): Metastasized lymph node, No PTEN expression, immunohistochemical stain for PTEN, 40X]



(L): Invasive ductal carcinoma, moderately differentiate strong PR nuclear staining (score 6+) [40X] (M) :Invasive ductal carcinoma, poorly differentiated , ER nuclear staining (score 7+)[40X].



(N): Invasive ductal carcinoma, strong membranous staining (score 3+) [immunohistochemical stain for HER-2/neu, 10X]

DISCUSSION

Continuous line of research that focuses on different forms of carcinogenesis underlining the growth of breast cancer has been established. In general, most of these projects were concerned with the possible correlations of various oncogenic, tumor suppressor genes (e.g. PTEN, p53), transcriptional factors (e.g. NFKB), adhesion molecules (e.g. ICAM1, 2 and 3) with various histopathological variables [11]. In this research, we were interested in studying the expression of PTEN because it had been become one of the most important gene in tumor biology. Its mutations, or dysregulation was found in many human tumors [12] and the Loss of PTEN activates the Akt pathway that was recognized to regulate multiple cellular processes, including apoptosis, proliferation, differentiation, and metabolism [13].It has newly been exhibited that Akt activation due to PTEN loss is associated with a worse prognosis among endocrine treated breast cancer patients [13].Accordingly, we work on the loss of PTEN expression with respect to hormone receptor status. Lipid Phosphatase activation involves dephosphorylation of phosphoinositide at the D3 position of the instill ring, and is reflect its action as a direct antagonist in the transmission system of PI3 kinase and PIP3 [14,15] The tumor-suppressing mechanism of PTEN had been incomplete understood through the Phosphatase function of PTEN. PTEN has dual-specific Phosphatase, this mean it had the role of a proteinphosphatase and a lipid Phosphatase.[16].Its role like a protein Phosphatase involve suppression of focal adhesion formation and cell invasion and migration by FAK dephosphorylation [16-18] . Thus, the current work represent a stride toward understanding the importance of PTEN as tumor suppressor gene during breast cancer growth, invasion, metastasis and recurrence, furthermore, immunohistochemical expression of PTEN and its correlation with other hormonal receptors status [PR ,ER ,HER-2] and other clinic pathological parameters in female breast cancer as being important in tumerogenesis, local invasion and metastasis. Immunohistochemical expression of PTEN in breast carcinoma is credible, as notified by Perren et al who [19] mentioned that immunohistochemistry was a powerful technique for determination of expression of PTEN protein as it provided with an internal control by staining of tumor tissue to that of the adjacent normal breast tissue [19]. In our study, PTEN expression loss had been detected in 48.3% of the cases. This result is in agreement with Chang et al. who found significant PTEN protein loss (48%) in breast cancer cases using immunohistochemical methods [20]. In addition, Park et a establish that PTEN expression loss in 35.6% of breast cancer tissues [21] and Bakarakos et al. found loss of PTEN protein in 72% women with a familial history of breast cancer [22]. By Perren et al. [19], decrease or no PTEN protein expression had been noted in 11(33%) of 33 breast carcinoma. Deposit et al. [23] had been detected a loss of PTEN protein expression in 73 (48%) of 151breast cancers, and Bose et al [9] detect that decrease PTEN protein expression in 13(38%) of 34invasive carcinoma of the breast. Shi et al. [24] found reduced or absence of PTEN expression in (36%) 28/77 cases of breast cancers. In manner which attract interest, loss of PTEN had been occurred more frequent in younger age (<50 year) at diagnosis. This explains the findings by Anders et al. [25]. That PTEN expression and genes involved in related signaling pathways had been altered in breast cancers that occurred in younger patients (≤ 45 years). We found that PTEN was absent in about 50.8% of ER / PR negative tumors. In comparison with others, Depowski, et al. [23] found that 68% of tumors that are negative for ER/PR, exhibit loss of PTEN expression Nevertheless, in our work, we didn't find a significant correlation between PTEN loss and ER/PR (P>0.05), however, we and Bose et al. found that there was no relation between loss of PTEN expression with the status of ER and

PR.[26] PTEN loss was seen in 57.1% of our HER-2 positive cases only. Since Pérez-Tenorio, found PTEN to sensitize breast cancers to targeted therapy with trastuzumab and consequently down-regulate the PI3K–Akt signaling pathway [27], this could be a factor that change the disease course and make the outcome better. A great association between PTEN protein loss and c-erb-B2 expression had not been reported [28] In this paper, PTEN loss was detected in 61.5% of TNBC cases and showed statistically significant correlation (P<0.05),Dean et al.[29] found that loss of PTEN expression had been detected in (48.3%) of patients with TNBC and associated significantly with younger age at the time diagnosis , Karseladze et al. [30]studied the expression of the PTEN gene product in TNBC by an immunohistochemical method, as well as detecting the gene by fluorescence in situ hybridization (FISH). The gene product appeared absent in 56 % of the tumor cell nuclei [30].we had been discovered that PTEN loss can be easily accessed by using immunohistochemistry. Our results reported PTEN expression was lost in (7.1%) in grade I compare with (14.3%) in grade II and (66.7%) of patient with grade III breast cancer, So there is significant difference among these grades (P<0.05) , this result agree with Park et al. [21] and Lee et al. [31],but disagrees with that reported by Depowski et al. (23). PTEN immunoexpression was recorded in (51.7%) of all cases ,it was positive in (45.1%) of stage I and negative in (6.9%), while it was positive in (29%)of stage II and negative in (17.3%), it was positive in (19.4%)of stage III and negative in (24.1%),it was positive in (6.5%)of stage IV and negative in (51.7%), So there is a significant difference among these stages (P<0.05). This finding agrees with that reported by Chang et al. [20] and Lee etal.2004 [31], but against what was suggested by Depowski et al.[23] Loss of PTEN expression is higher in breast cancer with lymph nodes metastasis than in lymph node negative cancer . In this study we have 44 cases (73.3%) out of 60 with lymph node metastasis , 35 out of 44 cases(79.5%) lost PTEN expression [7 out of 35 (20%) showed positive PTEN protein expression in breast mass but lost this expression in metastasized lymph nodes(altered expression)] ,and 9 out of 44 (20.5%) retained PTEN expression. Other 16(26.7%) out of 60 cases without lymph node metastasis ,this finding against what was suggested by Engine al.,(2006) [32], but agreed by Piekarski et al [33] Depowski et al. [23],and Lee et al. [31],pointed that loss of PTEN expression might be involved in stimulation of the invasive behavior of breast cancer.

CONCLUSIONS

- Loss of PTEN can readily assess using immunohistochemistry.
- There is a significant correlation between loss of PTEN expression with adverse prognostic factors of breast cancer including TNBC, high stages, high grades, and lymph nodes metastasis.
- There is no significant correlation between status of PTEN and age of the patients, histological types of breast carcinoma, progesterone, estrogen and HER-2 receptors.

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