

# The expression level of PTEN-gene is a diagnostic tool for gene alteration in invasive ductal carcinoma of the Breast

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## ABSTRACT

The phosphatase and tensin homolog gene are a tumor suppressor gene, and a key negatively regulator of cell signaling pathways that regulate growth and survival signaling pathways. Aim of study: Estimation the mPTEN in fresh breast cancer tissues versus apparently normal adjacent breast tissues in correlation with tumor marker Her/2 neu by immune histochemical staining.

**Patients and Methods:** A prospective study, from January 2013 to January 2015. Fifty-pairs of fresh tissues from both breast cancer of invasive ductal carcinoma "NOS" and apparently normal adjacent tissues (from modify radical mastectomy) were by patients were recruited at the Surgical Department/Al-Diawania Teaching Hospital in Al-Diawania city. Total RNA extraction and real-time quantitative polymerize chain reaction technique were used for assessment of PTEN gene expression. Tissue sample present in the paraffin embedded blocks belonging to tumor and normal adjacent tissue were used for histopathology examination and for immunohistochemical staining for Her/2 neu.

**Results:** All patients 50 (100%) exhibit PTEN gene expression down regulation, indicating fold change of cancer tissue for PTEN gene expression was significantly lower than that of normal adjacent tissue and the best cutoff value for gene expressional alteration in breast cancer tissues was (0.210) for diagnosis of gene alteration. Majority of cases (68%) were HER-2 negative.

**Conclusion:** gene expression of PTEN was significantly altered in breast cancer tissues. It has been no significant associated between PTEN gene expression and protein expression for Her/2.

**Keywords:** Invasive ductal carcinoma, mPTEN , RT-qPCR, Her/2, IHC.

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## INTRODUCTION

Overexpression of HER-2 has been recognized in about 10–34% of infiltrative breast cancers <sup>[1,2]</sup>, in 90% of these cases, where HER-2 gene amplification is present <sup>[1,3]</sup>. Some tumors with increased HER-2 mRNA and protein level have no detectable gene amplification. It is possible that alternative transcriptional and post-transcriptional mechanisms controlling. Beside it's prognostic value, HER-2 plays an important role in prediction of breast cancer outcome. Tumors with Positive HER-2 expression is associated with an optimistic response to anthracyclines, which may be explained by abundant simultaneous amplification with TOP2 $\alpha$  <sup>[4]</sup>. The phosphatase and tensin homolog (PTEN) gene are a tumor suppressor gene situated at 10q23 <sup>[5]</sup>. PTEN plays a major role in down regulation of cell growth and survival signaling pathways. Many studies have been published in which PTEN loss was correlated with resistance to herceptin and lapatinib in HER-2-neu positive breast carcinoma tissues and cell lines, because of HER-2 overexpression lead to hyper-phosphorylation of the phosphoinositide 3-kinase (PI3K) signaling pathway while PTEN loss leads to loss its function i.e dephosphorylation. Consequently, lead to PI3K pathway always active therefore PTEN loss have been found to predict poor prognosis and risk of progression following trastuzumab (alone or in combination with lapatinib) based therapy <sup>(5,6)</sup> , in one hand , and PTEN loss associated with poor response to tamoxifen therapy in HER-2 positive tumor <sup>[6,7]</sup> in another hand .The PTEN

normally inhibits the activation of PI3K by dephosphorylation of this path- way; then, PTEN lacking leads to continuous activation of PI3K/Akt pathway which is a major pathway for tumorigenesis .

## PATIENTS MATERIAL AND METHODS

The study was conducted during the period from January 2013 to January 2015. This is a prospective study, whereby patients were recruited at the Surgical Department/ AL-Diawania Teaching Hospital in Diawania city. Fifty patients diagnosed as having breast cancer who were subjected to the three principle evaluation methods: physical examination, imaging techniques (mammography and /or ultrasound) and (FNAC). Patients were analyzed for clinical data with special focusing on the age. Their ages range (30 -76) years with mean was (47+1.71) years, all cases underwent modify radical mastectomy and axillary clearance and no preoperative adjuvant chemotherapy or target therapy. Fifty-pairs of fresh tissues from both breast cancer of invasive ductal carcinoma and apparently normal adjacent tissues (NATs) which considered as healthy normal internal control , for total RNA extraction and for RT-qPCR were done in Veterinary medical college in Al-Diawania city. Another fifty pairs specimens of both breast cancer and NATs and referred to AL-Diawania Teaching Hospital for histo-pathological examination and after that for her/2neu IHC. The fresh tissue is preferable for RNA extraction and make it easier and earlier for molecular diagnosis than formalin fixed paraffin

embedded tissue (FFPET) , due to the cross linking of RNA with proteins, enzyme digestion happening during the procedure of fixation decreases the product, type and structure of RNA. Therefore, mRNA yield from stored paraffin block is minimal because of the labile property of mRNA and the harmful feature of enzymatic degradation

throughout the prolonged duration of archiving and RNA changes produced by fixation [8].

#### Probes and primers concerning mRNAs of PTEN and GAPDH gene

The mRNAs of PTEN and GAPDH gene probes and Primers has been made up by utilizing NCBI- Gene Bank data base [9]. The origin of primers is shown in table (1)

**Table 1:** The Primers and probes for mPTEN and mGAPDH gene

| Gene                                             | Sequence                          |                      |
|--------------------------------------------------|-----------------------------------|----------------------|
| Random Hexamer primer of PTEN and GAPDH for cDNA |                                   |                      |
| mPTEN primer                                     | F                                 | ACCAGTGGCACTGTTGTTTC |
|                                                  | R                                 | TTAGCTGGCAGACCACAAAC |
| mPTENprobe                                       | FAM-TGTTTCAGTGGCGGAACCTTGCA-TAMRA |                      |
| mGAPDH                                           | F                                 | TCAGCCGCATCTTCTTTTGC |
|                                                  | R                                 | TTAAAAGCAGCCCTGGTGAC |
| mGAPDH probe                                     | FAM-CCAGCCGAGCCACATCGCTC-TAMRA    |                      |

Tissue samples were homogenized in a denaturing lysis solution and dissolved RNA was stored at -20°C before use. Isolation of total RNA (RNA was extracted from fresh tissues using the Trizol reagent (Bioneer, Korea) according to the manufacturer's instructions. RNA quality was assessed with a NanoDrop 1000 spectrophotometer.

**Real-time RT-PCR for PTEN quantification:** mPTEN was evaluated according RT-kit procedure (Applied Biosystems, Foster City, CA, USA)" which involving use mPTEN-specific primer (according to mPTEN database to design the primers). Reverse transcriptase reactions was prepared to make cDNAs in a volume of 15 ml using, "10 ng total RNA for each sample, 50 nM stem-loop RT primer, 1 RT buffer, 1 mM each of dNTPs, 3.33 U/ml and 0.25 U/ml RNase inhibitor". Real-Time PCR was done in triplicate. The volume of 20 ml of each sample included, PCR Master Mix, 1 ml specific mPTEN Assay Mix, and 1.34 ml RT product. The reactions were incubated at 50 C° for 2 min and 95 C° for 10 min, followed by 40 cycles of 95 C° for 15 s and 60 C° for 1 min. All mPTEN quantification data were normalized to housekeeping gene. The messenger RNA(mRNA) of GAPDH gene primers and probe were designed by using NCBI- Gene Bank data base and Primer 3 plus design online .The cDNAs primer of GAPDH as design as Random Hexamer primer and the primer used in qPCR was: forward, 5-

UCCUUCAUUCCACCGAGUCUG-3 and reverse 5-GACUCCGGUGGAAUGAAGAAUU-3. Taq-Man probe for mGAPDH was: FAM-CCAGCCGAGCCACATCGCTC-TAMRA. The data results of RT-qPCR for mPTEN and GAPDH were analyzed by the relative quantification gene expression levels (fold change) were based on the Ct values by using the Livak method (Fold change =  $2^{-\Delta\Delta C_T}$ ) that described by (Livak and Schmittgen, 2001) [10].

#### Immunohistochemistry (IHC)

Tissue sections of about four to five micrometer thickness were adhered into positively enhanced slides in order to evaluate HER-2 status IHC. NATs (considered as internal control).

**(A)-Positive Control sections:** concomitant positive control tissue specimens have been run with each IHC procedure. These tissues were obtained from breast tumors already proved to be positive for HER-2.

**(B)- Negative Control sections:** these were obtained by technically omitting the primary antibody from the routine IHC procedure.

#### Immunohistochemical staining procedure

This was according to the protocol Streptavidin Biotin (LSAB+) as shown in kit [9].

#### Scoring system for HER-2/neu:

the reaction in Her/2 is membranous rather than nuclear [10]. As shown in table (2)

**Table 2:** IHC staining score concerning HER-2/neu

| Staining pattern                                                                                                                                   | Score | HER-2                               |
|----------------------------------------------------------------------------------------------------------------------------------------------------|-------|-------------------------------------|
| A strong complete membrane staining is observed in more than 30% (formerly 10%) of the tumor cells                                                 | 3+    | Strongly positive                   |
| A weak to moderate complete membrane staining is observed in more than 10% of the tumor cells                                                      | 2+    | Weakly positive (equivocal)need ISH |
| A faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane | 1+    | Negative                            |
| No staining is observed, or membrane staining is observed in less than 10% of the tumor cells                                                      | 0     | Negative                            |

**Statistical analysis:** SPSS version 16 and Microsoft Office Excel 2007 were using in analysis of these data; Chi-square test and Fisher exact test were used to study association between any two nominal variables. P-value of less than or equal to 0.05 was considered significant.

## RESULTS

### Human epidermal growth factor receptor-2 protein expression

Patients with positive IHC HER-2 expression accounted for 16 (32%) only. Patients with score +3 represented a

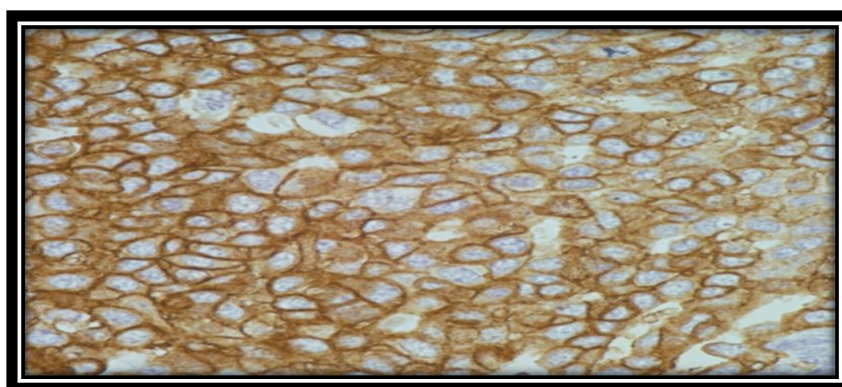
large proportion, 10 (20%), while patients with score+2 accounted for 6 (12%) which consider as equivocal cases,

as shown in table (3). The HER-2 IHC illustrated as figures (1) and (2).

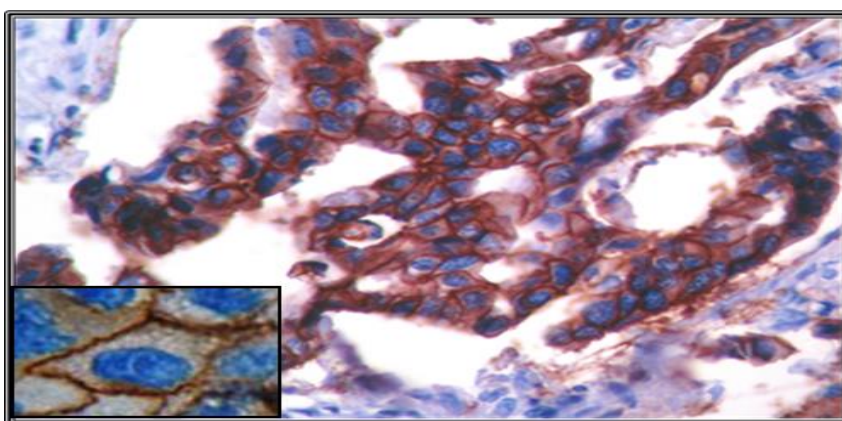
**Table 3:** IHC for HER-2 expression in patients with breast carcinoma

| HER-2/neu score* | No. | %   |
|------------------|-----|-----|
| 0 (-ve)          | 32  | 64  |
| 1 (-ve)          | 2   | 4   |
| 2 (+ve)          | 6   | 12  |
| 3 (+ve)          | 10  | 20  |
| Total            | 50  | 100 |

\*Patients with score 0 and score 1 were regarded as negative, while patients with score 2 and 3 were regarded as positive.



**Figure 1:** Breast, invasive ductal carcinoma. HER-2/neu positive score +2. Arrow shown complete membrane staining with weak - moderate brown discoloration was observed in more than 10% of the tumor cells, (20X).



**Figure 2:** Breast, invasive ductal carcinoma. HER-2/neu positive score +3. Arrow inside inset shown complete membrane staining with strong brown discoloration was observed in more than 30% (formerly 10%) of the tumor cells with fish net appearance, 20X.

#### PTEN- gene expression fold change

Mean cancer tissue fold change of mPTEN gene was significantly lower than that of NATs ,0.285±0.041 versus

0.859±0.077, respectively (P<0.001). These results are detailed in table (4).

**Table (4):** Comparison of mean gene fold change between breast cancer tissues and NAT

| Parameter | Group  | Median | Mean  | SE    | Minimum | Maximum | P      |
|-----------|--------|--------|-------|-------|---------|---------|--------|
| mPTEN     | Normal | 0.811  | 0.859 | 0.077 | 0.233   | 2.457   | <0.001 |
|           | Cancer | 0.176  | 0.285 | 0.041 | 0.022   | 0.967   |        |

All patients exhibit mPTEN gene down regulation, as in table (5).

**Table 5:** The mPTEN gene expression level.

|                 | mPTEN gene |     |
|-----------------|------------|-----|
|                 | No.        | %   |
| Down regulation | 50         | 100 |
| Up regulation   | 0          | 0   |
| Total           | 50         | 100 |

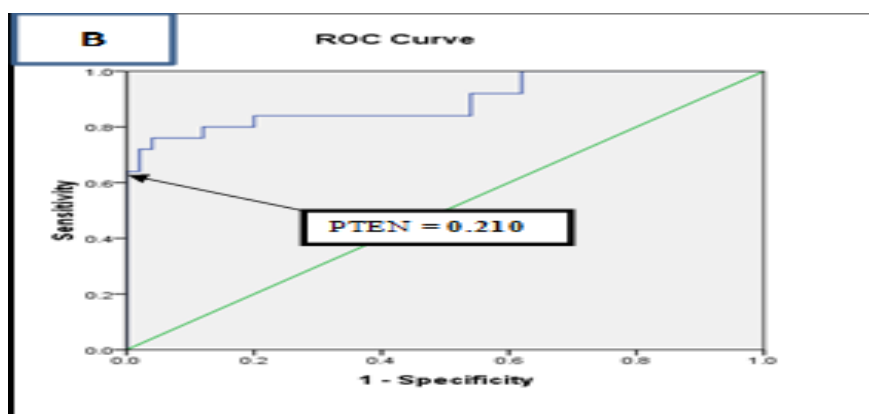
#### 7-Validity of PTEN-gene expression fold change as gene alteration

To find the cutoff value for PTEN gene expression fold change that predict gene expression alteration in breast

carcinoma by using the RT-qPCR technique , an ROC curve analysis was done that showed the following results: The best cutoff value for PTEN was 0.210 as shown in table (6) and figure (3).

**Table 6:** Validity of PTEN gene expression fold change in predicting gene expression alteration in breast cancer tissues.

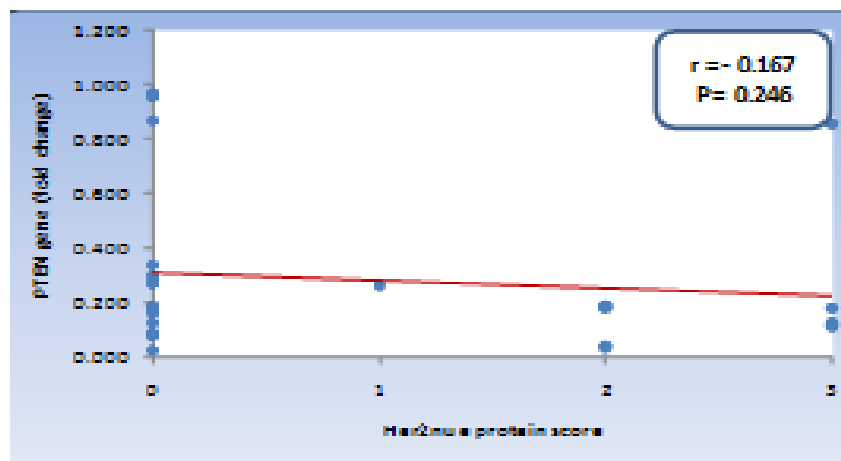
|                | PTEN   |
|----------------|--------|
| Cutoff value   | 0.210  |
| Accuracy (AUC) | 0.891  |
| P-value        | <0.001 |
| Sensitivity    | 64%    |
| Specificity    | 100%   |
| Interpretation | Good   |



**Figure 3:** mPTEN fold change that predicts gene alteration in breast cancer tissues (B); with p value was (<0.05) .

#### Correlation between Human epidermal growth factor receptor-2 expression and fold change of mPTEN

mPTEN gene fold change showed a negative non-significant correlation with IHC HER-2 expression as shown in figure (4).



**Figure 4:** Correlation between HER-2 protein expression and fold change of mPTEN.

Table 7: Validity of mPTEN gene expression as a prognostic marker

| Prognostic parameter | mPTEN Cutoff value | AUC (accuracy) | Specificity | Sensitivity | P-value | Interpretation |
|----------------------|--------------------|----------------|-------------|-------------|---------|----------------|
| +ve HER-2/neu        | ≤0.140             | 0.630 (63%)    | 73.5%       | 50%         | >0.05   | Poor           |

## DISCUSSION

Concerning to result of IHC for HER-2 protein, majority of cases were HER-2 negative ,68% and cases with HER-2 positive 32% only. These results were different with that results which conducted by (Gamber) [11], in which majority of cases were positive HER-2, 63.2% while negative cases for HER-2 were ,36.8%. The differences could be due to the presented study was prospective study and (Gamber) [11] study was retrospective studies , while these results accepted with that result of those reported by (Hong *et al*) [12] whom found that the positive HER-2 were 26.7%. In the present study , all patients 50(100%) exhibit PTEN mRNA down regulation and the mean cancer tissue fold change of PTEN gene expression was significantly lower than that of NATs , its expression levels was (0.576) folds lower than the samples having NATs. These findings are disagreement with other study which reported by (Irina *et al*) [13], in which this study consider as first study to be evaluate the PTEN gene expression on the level of mRNA by RT-qPCR using (SYBR green) in both breast cancer and apparent NATs ,since 2013 , and no others studies which evaluate the PTEN gene expression in both breast cancer and apparent NATs by RT- qPCR using (Taq-Man Probe). It was found that the majority of cases of IDC (80%) showed up-regulation of PTEN mRNA in cancer tissues in comparison to NATs and PTEN gene expression was reduced in cancer tissue only in 20% of cases.

This discrepancy between the results of present study from that of Irina study, which used in their study the paired material, both breast carcinoma and NAT from (lumpectomy mass). Furthermore, about 15% of their tissues sample were unavailable due to insufficient amount of NATs for quality of RNA and for q-PCR analysis and because the loss of these data, it suggest to bias their results (Irina *et al*) [13]. In the other hand, most others studies used IHC for protein expression of PTEN gene in cancer tissues of IDC ,but these studies did not evaluate PTEN gene expression in comparison with the corresponding NATs .These studies that used IHC for PTEN protein expression demonstrated that loss of PTEN expression in 30-50% of IDC (Hong *et al*) [7,22]; (Engin *et al*) [14,20] and (Osamu *et al*) [1,18]. While, the result of present study used RT-qPCR Taq-Man probe to determine expression of PTEN gene, which enables quantification relative to a housekeeping gene and is not biased by subjective factors such as the experience of the assessor like IHC (Tvrdik *et al*) [16,21] and (Menndoza *et al*) [17,19]. In the present study, there was no statistically significant correlation between HER-2 status as protein level and PTEN gene expression. These findings were in accepted with other studies which reported by (Hong *et al*) [12].

## CONCLUSION AND RECOMMENDATIONS

The quantitative RT-PCR is cost effective method to evaluate gene expression. The PTEN gene expression is significantly downregulated in breast cancer tissues of invasive ductal carcinoma, PTEN gene as a candidate

tumor suppressor gene. Down regulation of PTEN gene expression in breast cancer tissues of invasive ductal carcinoma were not significantly correlate with HER-2 by IHC technique. Evaluation of PTEN gene expression in serum and other body fluid like (saliva) of breast cancer patients which considered as non- invasive technique before and after operation, and before and after gene therapy for follow up patient with breast cancer.

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