REVIEW ARTICLE

The Phosphatase And Tensin Homolog-Gene (PTEN-Gene) Expression Level is a Novel Indicator for Poor Prognosis in Invasive Ductal Carcinoma

Shoroq Mohammed AL-Temimi[2], Adel Mosa AL-Rekabi[1]*

[1]*Department of Pathology, College of Medicine, University of Al-Qadisiyah, Al Diwaniyah, Iraq*
[2]*Department of Surgery, College of Medicine, University of Al-Qadisiyah., Al Diwaniyah, Iraq*

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ABSTRACT

The objective of the present work was to formulate and evaluate floating microspheres containing Roxatidine acetate HCl prepared by emulsion solvent diffusion technique. To achieve extended retention in the upper gastrointestinal tract (GIT), this may result in enhanced absorption and thereby improved bioavailability. Roxatidine acetate HCl is a competitive H_2 receptor antagonist used to treat gastric ulcers. Floating microspheres systems have a density less than gastric fluids, and so these systems remain buoyant in the stomach without affecting the gastric emptying rate for a prolonged period. Floating microspheres were prepared by using hydroxy propyl methyl cellulose (HPMC K4M and HPMC K15M) and ethyl cellulose. The microspheres were evaluated for parameters like particle size, entrapment efficiency, shape and surface morphology, drug content, *in-vitro* drug release study and drug release kinetic study. The floating microspheres were found to be spherical, and the FTIR study confirmed the drug-polymer compatibility. All floating microspheres formulations showed good flow properties, and buoyancy was found to be 71–87%. Among all formulations, F2 showed an appropriate balance between buoyancy and drug release rate (90% in 12 hour); hence it is considered as the best formulation. The data obtained in this study thus suggests that the floating microspheres of roxatidine acetate HCl are promising for sustained drug delivery, which can be used for reducing dosing frequency.

Keywords: Buoyancy, Drug release, Floating microspheres, Roxatidine.

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INTRODUCTION

Breast cancer is a very complex multifactorial disease caused by interactions of both hereditary and environmental factors, leading to a gradual accumulation of genetic and epigenetic alterations in malignant breast cells.[1]

The phosphatase and tensin homolog (PTEN) gene is a tumor suppressor gene situated at 10q23.[2] PTEN plays a major role in the 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 lose its function i.e dephosphorylation.[2,3] 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,[2,3] in one hand and PTEN loss associated with poor response to tamoxifen therapy in HER-2 positive tumor,[3,4] 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 age. Their ages range (30-76) years with mean was (47 + 1.71) years; all cases underwent modified 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 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

**Author for Correspondence: adel.ahmed@qu.edu.iq*

specimens of both breast cancer and non-accidental trauma (NATs) and referred to AL-Diawania Teaching Hospital for histopathological examination. The fresh tissue is preferable for RNA extraction and makes 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.[5]

Probes and primers concerning mRNAs of PTEN and GAPDH gene

The mRNAs of PTEN and GAPDH gene probes and primers have been made up by utilizing NCBI- Gene Bank database.[6] The origin of primers is shown in Table 1.

MiRNA isolation from tissue

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 manufacturers' instructions. The RNA quality was assessed with a NanoDrop 1000 spectrophotometer.

Real-time RT-PCR for PTEN quantification

mPTEN was evaluated according to RT-kit procedure (Applied Biosystems, Foster City, CA, USA)," which involved 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 the housekeeping gene. The messenger RNA(mRNA) of GAPDH gene primers and probe were designed by using NCBI- Gene Bank database 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-UCCUUCAUUCCACCGGAGUCUG-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 = $2^{-\Delta\Delta CT}$) that described by (Livak and Schmittgen, 2001).[8]

Statistical Analysis

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

RESULTS

PTEN- gene expression fold change

Mean cancer tissue fold change of the 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 2.

All patients exhibit mPTEN gene downregulation, as in Table 3.

Correlation between fold change of PTEN- gene expression and age of the patients.

Being numeric variables, age, and fold change can be related using Pearson's correlation coefficient statistical tool. Despite

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

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

Table 2: 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	

Table 3: The mPTEN gene expression level.

	mPTEN gene	
	No.	*%*
Downregulation	50	100
Upregulation	0	0
Total	50	100

a positive correlation with age, exhibited by mPTEN gene, there was no statistical significance. Figure 1 outlined these correlations.

Mean fold change of mPTEN in patients <50 years was not statistically significantly different from that of patients ≥50 years (0.265 ±0.054) versus (0.310 + 0.063), respectively. These correlations are outlined in Figure 2.

Correlation between the size of tumor and fold change of mPTEN

The mPTEN gene fold change showed a negative correlation with size of tumor. Again this correlation was not statistically significant. Figure 3 illustrate these correlations.

Correlation between fold change and lymph node metastasis

Mean mPTEN fold change of patients with positive lymph node was significantly lower from that of patients with negative lymph node involvement, 0.125 + 0.010 versus 0.525 + 0.073, respectively, and p-value was <0.001. Figure 4 shows these results.

A significant negative correlation between mPTEN gene fold change and involved lymph nodes. As shown in Figure 5.

Correlation between grade of tumor and fold change

When grade was plotted against mPTEN fold change, a negative correlation, it did not reach a statistical significance, r = –0.091 and p = 0.532. As shown in Figure 6.

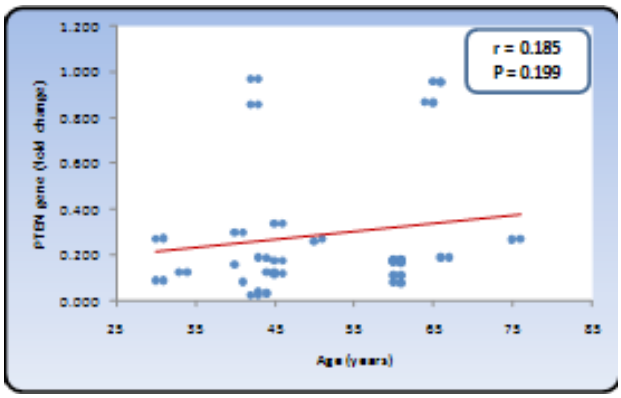

Figure 1: Correlation between fold change, of mPTEN gene, and age

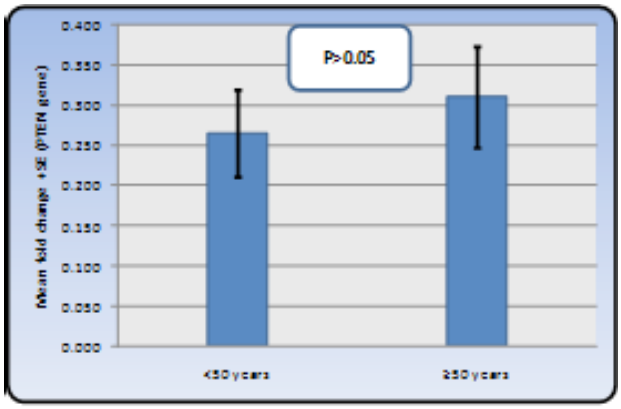

Figure 2: Correlation of mean fold change of PTEN patients with <50 years and ≥ 50 years.

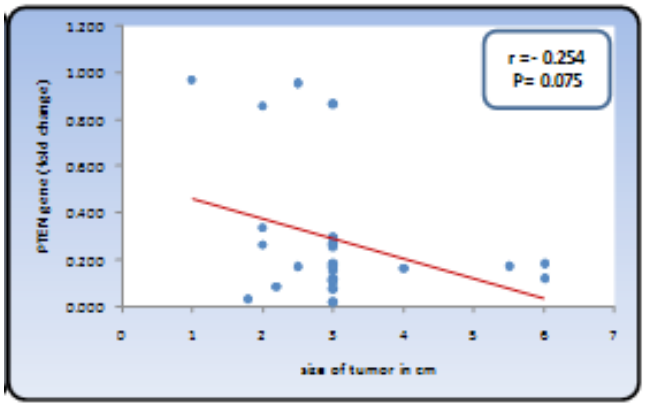

Figure 3: Correlation between size of tumor and fold change mPTEN.

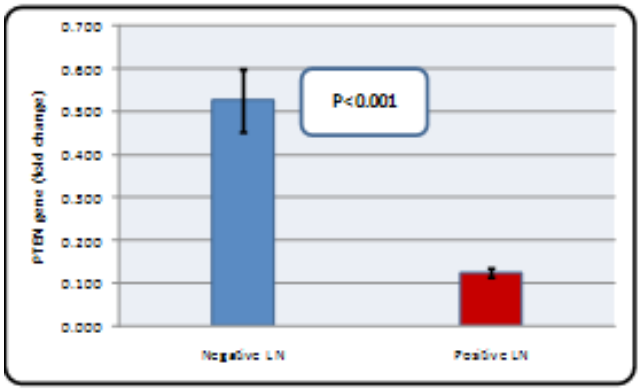

Figure 4: Correlation of mean fold change of mPTEN between patients with positive lymph nodes and patients with negative lymph nodes

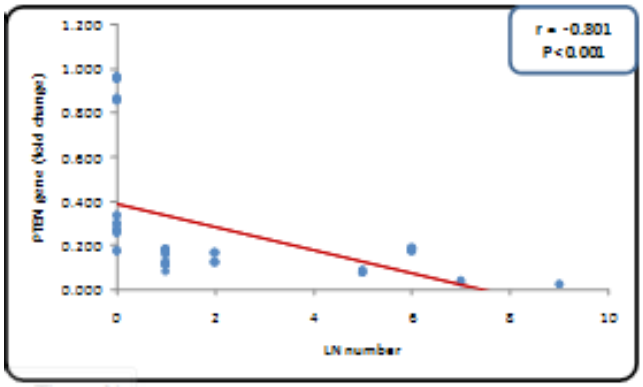

Figure 5: Correlation between number of involved lymph nodes and mPTEN fold change.

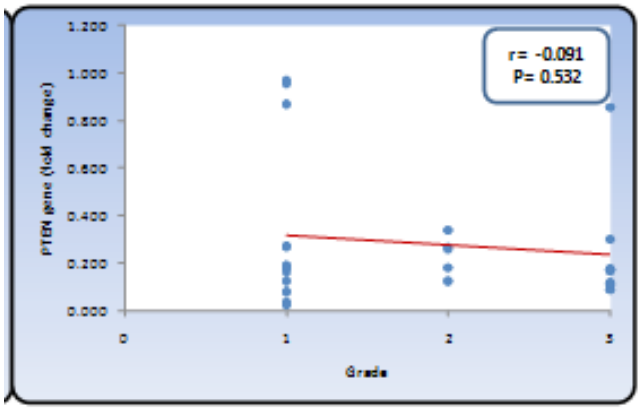

Figure 6: Correlation between fold change and grade of the tumor.

Correlation Between Stage and Fold Change

There was a significant negative correlation between stage and mPTEN gene fold change. These results are shown in Figure 7.

Validity of PTEN-Gene Expression Fold Change as Gene Alteration

To find the cutoff value for PTEN gene expression fold change predicting the gene expression alteration in breast carcinoma. By using the RT-qPCR technique, a ROC curve analysis was done that showed the following results: The best cutoff value for PTEN was 0.210, as shown in Table 4.

To find the cutoff value of fold change for PTEN gene expression, that predicts breast cancer patient with positive lymph node metastasis and patients with AJCC stage III, IV (higher stage) breast cancer from patients with earlier stages of breast cancer, an ROC curve analysis was performed.

The result of PTEN gene expression fold change can predict positive lymph node and higher stage (III, IV). The ROC results demonstrated that the AUC was (98%, excellent), (81.1%, good), when the cutoff value was set to the optimal point, (≤0.175), (≤0.098); specificity was (100%), (93.1%); sensitivity was (80%), (50%) respectively and (p-value was <0.05). Results obtained were summarized and presented in Rable 5.

DISCUSSION

The present study conducted in Iraq, evaluating PTEN gene expression by RT-qPCR, in the same series of fresh breast cancer/apparently NAT samples in a sample of Iraqi female patients. There was no baseline study regarding PTEN gene expression stratification in apparently NATs in Iraqi individuals. A potential limitation in this study was the limited number of patients and also the need for fresh tissue of breast cancer and NATs for RT-PCR analysis. Subsequently, it depended upon the modified radical mastectomy specimen only.

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 were (0.576) folds lower than the samples having NATs. These findings are disagreement with other studies which reported by (Irina *et al.*),[9] in which this study considers as the first study to 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 the present study and 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.*)[9] While 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.*);[4] (Engin *et al.*)[10] and (Osamu *et al.*).[11] While, the result of present study used RT-qPCR Taq-Man probe to determine the 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.*[12] and Menndoza *et al.*).[13]

In the present study, there is no statistical significance between age of patients and fold change of PTEN gene expression. These findings are in agreement with (Hong *et al.*),[4] (Irina *et al.*),[9] (Engin *et al.*),[12] and (Osamu *et al.*).[11]

According to PTEN mRNA, there was no statistically significant correlation between PTEN gene expression and tumor size. These findings were in agreement with other

Table 4: 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

8-Validity of PTEN- gene expression fold change as a prognostic marker.

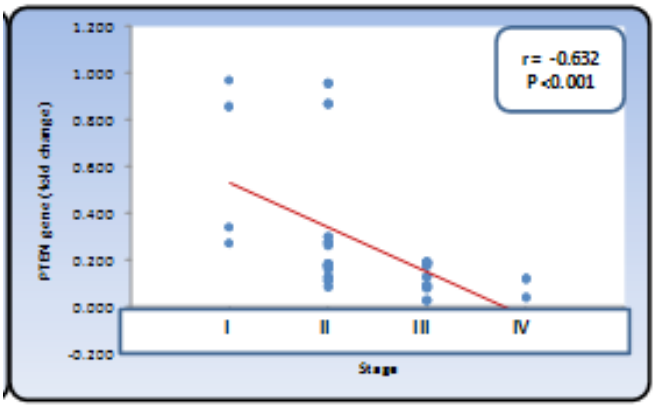

Figure 7: Correlation between stage and fold change

Table 5: Validity of mPTEN gene expression as a prognostic marker

Prognostic parameter	*mPTEN Cutoff value*	*AUC (accuracy)*	*Specificity*	*Sensitivity*	*p-value*	*Interpretation*
Positive LN	≤ 0.175	0.980 (98%)	100%	80%	< 0.05	Excellent
Size ≥ 2 cm	≤ 0.961	0.522 (52.2%)	50%	100%	> 0.05	Poor
Stage (III, IV)	≤ 0.098	0.811 (81.1 %)	93.1%	50%	< 0.05	Good
Grade (II, III)	≤ 0.172	0.524 (52.45)	67.5%	50%	> 0.05	Poor

studies reported by (Engin *et al*.),[10] who found that no statistically significant correlation found between loss protein expression of PTEN and the tumor size. While disagreement with other studies which reported by (Hong *et al*.),[4] (Osamu *et al*.)[11] and (Depowski *et al*.),[15] who found that, a negative correlation and statistically significant correlation between loss protein expression of PTEN and the tumor size.

Mean PTEN gene expression was significantly lower in the positive L.N involvement; its expression levels was (0.4) folds lower than the samples having negative lymph node involvement. These findings were in agreement with other studies reported by (Depowski *et al*.)[15] and (Osamu *et al*.),[11] who found that a negative correlation and statistically significant between loss protein expression of PTEN and positive lymph node involvement. While different from other studies reported by (Hong *et al*.),[4] (Irina *et al*.)[2] and (Engin *et al*.)[10,16] who found that, no statistically significant between loss protein expression of PTEN and L.N involvement.

Mean PTEN gene expression was significantly lower in stage III and IV compared to stage I and II. These findings were accepted with other studies reported by (Hong *et al*.)[4] and (Osamu *et al*.),[11] who found that a negative correlation and statistically significant between loss protein expression of PTEN and higher stage. While disagreement with other study reported by (Depowski *et al*.),[15,17] who found that, no statistically significant between loss protein expression of PTEN and higher stage.

In the present study, there was no statistically significant correlation with tumor grade and PTEN gene expression. Regarding the PTEN gene expression, the results of the present study were accepted with other studies reported by (Engin *et al*.)[10,18,19] and (Depowski *et al*.).[15,20,21]

CONCLUSION

The PTEN gene expression is significantly down-regulated in breast cancer tissues of invasive ductal carcinoma, PTEN gene as a candidate tumor suppressor gene, and downregulation of PTEN gene expression has been associated with positive lymph node involvement and higher tumor stage (III, VI). Downregulation of PTEN gene expression in breast cancer tissues of invasive ductal carcinoma were not significantly correlate with other patients' criteria like (age, grade, and size of the tumor).

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