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Immunohistochemical assessment of PTEN expression and its association with tamoxifen resistance in ER positive breast cancers

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ABSTRACT

Introduction: Although Estrogen Receptor (ER) positivity is a good prognostic factor in breast carcinoma (BC), a subset of patients experiences poor disease-free survival (DFS). Mutation in phosphatase and tensin homologue deleted on chromosome ten (PTEN) is identified as a poor prognostic feature in BC. This study was designed to find out the impact of lost or poor PTEN expression on ER positive BC, in terms of the recognized prognostic factors and survival outcome to find out its association with tamoxifen resistance.

Methods: This was a cross sectional study with a follow up component. BC tissue blocks submitted to our unit from 2006 to 2012 were selected. From the laboratory data, patients who had ER positive BC, undergone mastectomy, treated with tamoxifen were selected. All clinicopathological parameters, DFS and overall survival (OS) were analysed against lost or poor PTEN expression. Clinicopathological features were compared using Chi-square test. Kaplan-Meier model with log-rank test was used for the survival analysis.

Results: A total of 130 BC patients satisfied the inclusion criteria. PTEN expression was lost or poor in 82.3% (n=107) patients. PTEN expression had a positive association with the level of ER expression (p=0.011) and a negative association with Nottingham prognostic index (NPI) (p=0.045) and pathological stage (p<0.048). Only 12.1% (n=16) patients had recurrences and 7.69% (n=10) had died over a period of 51 months of mean follow up. There was no significant association between PTEN expression and survival.

Conclusions: This study showed that there is a statistically significant association between lost or poor PTEN expression and low ER expression, high NPI and stage 3 in ER positive BC. Further studies including larger study sample with a longer follow up are recommended to find out the association of PTEN with the survival in ER positive BC treated with tamoxifen.

Key words: *Breast carcinoma, ER, Immunohistochemistry, PTEN*

Introduction

Breast cancer is the second commonest cancer in the world and the most frequently occurring cancer among females (1). In the year 2012, 1.67 million new breast cancer cases were diagnosed and that is about 25% of all cancers around the globe (1). It is also the fifth leading cause of death of all cancers (1). Breast cancer is the commonest cause of cancer death in women in underdeveloped countries and the second commonest cause of cancer death in more developed regions in the world (1). In Sri Lanka, it is the leading cancer among females and accounts for 25.4% of diagnosed cancer among females (2). It also accounts for the highest cancer mortality in Sri Lankan females (2, 3).

Tamoxifen is the most commonly used selective estrogen receptor modulator (SERM) which is used for the treatment and prevention of estrogen receptor (ER) positive breast cancer and it has been the first line treatment for premenopausal patients with ER positive breast carcinoma (4). Tamoxifen acts as an anti-estrogen agent in the breast tissue. It acts by binding to ER leading to a conformational change in the receptors. This results in blockage in the expression of estrogen dependent genes. The prolonged binding of tamoxifen to the nuclear chromatin leads to decreased estrogen response by tumour cells, hence growth arrest and induction of apoptosis within the breast cancer cells takes place (5).

PTEN, also known as MMAC1 (mutated in multiple advanced cancers), is a tumour suppressor gene located at chromosome 10q23. PTEN mutation is associated with tumorigenesis, cancer progression and drug resistance and it is the second most frequently mutated gene in human cancer after p53 (6). Varieties of human tumours are known to associate with PTEN mutation, which includes glioblastoma, prostatic carcinoma, endometrial carcinoma, breast carcinoma and melanoma. Germline mutations in PTEN gene are known to cause Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome (PTEN hamartoma tumour syndrome) characterised by a high risk of cancers including breast cancer. Affected female patients with CS syndrome have 25% - 50% life time risk of developing a breast carcinoma. Around 30% - 40% of sporadic breast carcinomas show PTEN loss (7).

PTEN acts as a tumour suppressor by antagonizing the phosphatidylinositol (4,5)-triphosphate kinase (PI3K)/ proteinkinase B (Akt) signaling pathway by dephosphorylating phosphorinositol 3,4,5-triphosphate (PIP3), a key signaling component of PI3K/Akt pathway and thereby modulating cell cycle progression and cell survival. The biological consequences of inhibition of PI3K/Akt pathway include stimulation of apoptosis and inhibition of cell cycle entry by halting G1 to S phase progression leading to growth inhibition (8). Therefore, mutation or reduced expression of PTEN can lead to inhibition of tamoxifen induced apoptosis leading to tamoxifen resistance in PTEN mutated breast carcinoma.

Though many studies have been done in the past to identify the role of PTEN gene mutation in various cancers, its prognostic significance in breast cancer is not sufficiently investigated. A few studies have found out that reduced PTEN expression in breast cancer have a significant relationship with tumour size, pathological stage, lymph node metastases and ER and Progesterone Receptor (PR) status (9, 10).

The aim of the study was to investigate the role of PTEN gene as a prognostic marker in ER positive breast cancer patients by analysing immunohistochemical expression of PTEN and its association with recurrence of disease, survival, stage, grade, tumour size and hormonal receptor status and to find out its association with tamoxifen resistance.

Methods

This was a cross sectional study with a follow up component which included 130 breast cancer patients. Breast cancer tissue blocks submitted to our unit from 2006 to 2012 were selected. Using data from the laboratory database and co-investigator's database, patients who had ER positive BC, undergone mastectomy, treated with neo-adjuvant tamoxifen therapy were selected. Wax blocks with perished tissue, haematoxylin and eosin (H&E) slides showing autolytic changes and patients who were stage IV at presentation were excluded from the study. All relevant clinical parameters were retrieved from the histopathology reports at the Department of Pathology, Faculty of Medicine, University of Ruhuna and the survival data were retrieved from the co-investigator's data base.

Following definitions were used to define Recurrence Free Survival (RFS) and Overall Survival (OS) which are the same definitions that were used to define the above endpoints in the said data base that contains patients follow up details.

Recurrence free survival (RFS) - Time from the date of diagnosis to the date of confirmation of development of local, regional and/ or distant recurrences (11).

Overall survival (OS) - Time from the date of diagnosis to the date of death due to any reason (11).

Date of diagnosis of the disease - Date of diagnosis or confirmation of breast carcinoma by Fine Needle Aspiration Cytology (FNAC), tru cut, and incision or excision biopsy; whichever was done first.

Date of recurrence - Date of diagnosis of recurrence by histology, cytology or radiology; whichever was done first.

Tissue microarray (TMA) blocks were prepared from the wax blocks with breast cancer tissue for the PTEN assessment. Normal breast tissue was taken as the control.

Immunohistochemistry

PTEN immunohistochemistry was done manually with anti-PTEN antibody (monoclonal, mouse anti human, clone 6H2.1, dilution 1:100, Dako) with EnVision system (HRP labeled Polymer, Dako) and chromogen Dako Dab liquid. Immuno-histochemistry staining was performed according to the protocol which was optimised and validated for PTEN, in our laboratory.

The sections were taken on to poly-L-Lysine coated slides and were incubated overnight at a temperature of 60ºC. Then the slides were deparaffinized and hydrated by passing through Xylene and graded series of alcohol. Antigen retrieval was performed by pressure cooking in pH 9 buffer. Then the sections were treated with endogenous peroxidase blocking buffer for 15 minutes to block the endogenous peroxidase activity. As the next step, sections were incubated overnight in the humidified chamber with 6H 2.1 PTEN primary antibody (dilution 1 : 100). Afterwards, they were washed in PBS buffer twice and treated with the secondary antibody (EnVision system). After washing, Dab substrate buffer solution (freshly made) was added to the sections to reveal the PTEN antibody. Following this step, the sections were again washed with PBS buffer and counterstained with Harris Haematoxyline and differentiated with acid alcohol and mounted with DPX.

Interpretation of Staining for PTEN

PTEN immunohistochemical expression can show cytoplasmic and/or nuclear localization (12). In our study it was predominantly cytoplasmic and normal glandular epithelium was taken as the control as it shows immunoreactivity for PTEN. Duct epithelial cells and myoepithelial cells showed strong cytoplasmic staining for PTEN. Stromal cells and inflammatory cells also showed strong cytoplasmic staining for PTEN which were useful as internal controls.

Scoring for PTEN immune-expression was done according to a semi-quantitative scale, introduced by Andrade *et al.,* which is based on intensity of immunohistochemical staining (12). According to the intensity of staining, the tumours were divided in to three groups. Staining intensity of normal duct epithelial cells was taken as the control (Figure 1). The group assigned as "0" had no staining (Figure 2a), group assigned as +1 had reduced staining (Figure 2b) and group assigned as +2 had equal staining intensity (Figure 2c), compared to normal duct epithelial cells (Table 1).

Table 1: Scoring of PTEN immune expression by tumour cells

PTEN expression (compared to normal duct epithelial cells) by tumour cells	Score
No staining	0
Reduced staining intensity	+1
Equal staining intensity	+2

Statistical analysis

Statistical analysis was done by using *SPSS20* software. Chi-square test was used to determine the associations between different variables. Recurrence free survival and Overall survival were calculated by the Kaplan-Meier survival estimates and log rank test. The level of significance was set at 0.05.

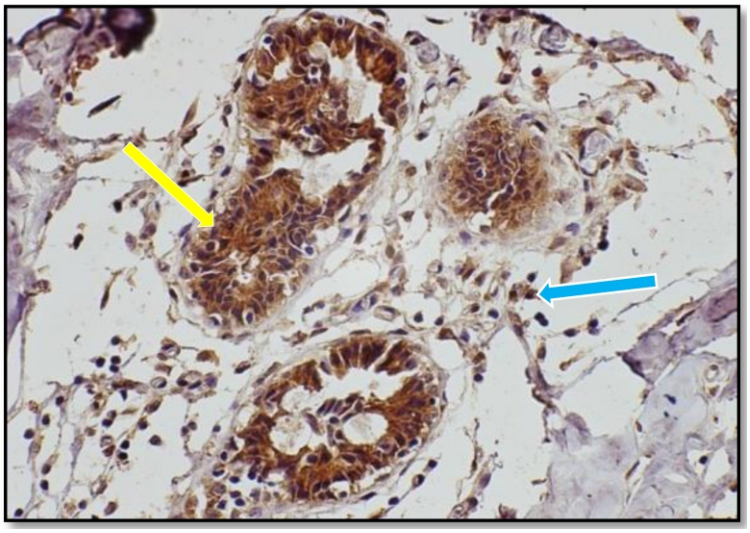

Figure 1: Normal breast tissue showing strong cytoplasmic positivity for PTEN (yellow arrow). Background inflammatory cells also show cytoplasmic positivity for PTEN (blue arrow), which was useful as an internal control. (H&E x 40)

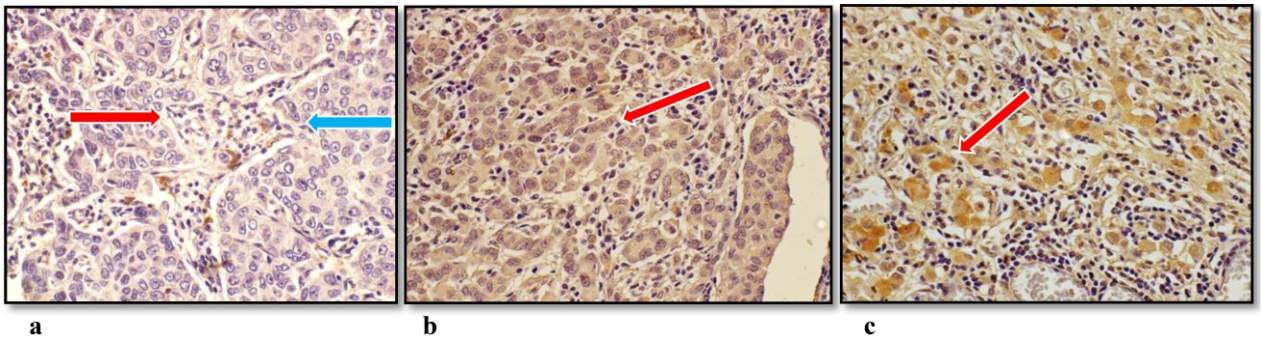

Figure 2: a. Score 0 = staining undetectable in tumour cells (red arrow). Background inflammatory cells show cytoplasmic positivity (blue arrow),
b. Score 1 = staining weaker than normal duct epithelial cells (red arrow),
c. Score 2 = staining equal to that of normal duct epithelial cells (red arrow) (H&E x 40)

Results

Clinicopathological findings

The study sample consisted of 130 patients with ER positive breast cancer who had underwent modified radical mastectomy. Majority of the patients were between ages 36 and 60 years (71%) and the mean age at diagnosis was 53 years. All patients have been treated with standard adjuvant tamoxifen therapy. Mean patient follow-up period was 51 months (6-93 months). At the completion of the study, 10 patients had died and 120 were alive. In the study group, 117 (90%) patients had duct carcinomas while 10 (7.69%) patients had lobular carcinomas and only 3 (2.3%) patients had mucinous carcinomas. Out of the total number of 130 patients, lymph node (LN) metastasis was present in 69 (54.3%), whereas only 58 (45.7%) were nodes negative. Disease recurrence occurred in 16 (12.1%) of patients while 113 (86.9%) had no recurrence at the end of the follow up. Out of those who had recurrences, 14 (10.8%) had metastasis and two had local recurrence (1.5%). Patients' characteristics and tumour characteristics are shown in Table 2.

Table 2: Patients' characteristics

Characteristics	Number	Percentage
Tumour size		
< 2 mm	55	44%
2 - 5 mm	64	51%
> 5mm	7	5%
Histological type		
Duct	113	90%
Lobular	10	8%
Other	7	2%
Nottingham Grade (NG)		
1	27	21%
2	75	58%
3	26	20%
Poor fixation	2	1%
Nottingham prognostic index (NPI)		
< 3.4	30	25%
3.4 - 5.4	72	59%
> 5.4	20	16%
Lymph node stage		
1	111	78%
2	16	12%
3	10	8%
Pathological stage		
I	28	22%
II	64	52%
III	32	26%

ER, PR and Her2 phenotype

All patients were ER positive (100%) and Her 2 negative (100%) while 113 (87.6%) patients were PR positive.

PTEN immunophenotype

PTEN expression was positive (Score 2+) in 23 (17.7%) patients while it was negative (Score +1 or Score 0) in 107 (82.3%) patients. PTEN immuno-expression was analysed in relation to clinicopathological parameters. No correlation was found between PTEN expression with patients age category (p=0.301), tumour size (p=0.178), histological type, lympho-vascular invasion (p=0.232), Nottingham grade (p=0.46), LN metastasis (p=0.106), PR expression (PR=0.127), recurrence of the disease (p=0.304). However, 26/27 (95.7%) in the low ER expression category (Allred score 3 and 4) was PTEN negative, while PTEN negativity was observed in 81/103 (78.6%) patients in ER high expression category (Allred score 5 to 8). Therefore, PTEN negativity was more frequent in tumours with low ER expression (p=0.023) demonstrating a strong positive association of PTEN expression with low and high ER expression. In contrast, PTEN negativity was frequent among tumours with high NPI score than low NPI score; 22/23 (95.7%) tumours with a high NPI score (>5.4) were PTEN negative, whereas only 78/99 (78.8%) tumours in the low NPI score category had PTEN negativity. These results exhibit a negative association of PTEN expression with NPI score (p=0.045). Similarly, PTEN negativity was significantly more frequent in stage 3 tumours than stage 1 and 2 (30/32vs72/92) p = 0.0480. This also highlights the negative association of PTEN expression with pathological stage of the tumour.

Survival analysis

PTEN expression was analysed against overall survival (p=0.713) (Figure 3a) and recurrence free survival (p=0.452) (Figure 3b) and failed to demonstrate a significant association. Survival analysis in relation to PTEN expression was also done in separate groups of patients according to LN stage, pathological stage, ER expression and NPI value, but failed to demonstrate a significant relationship.

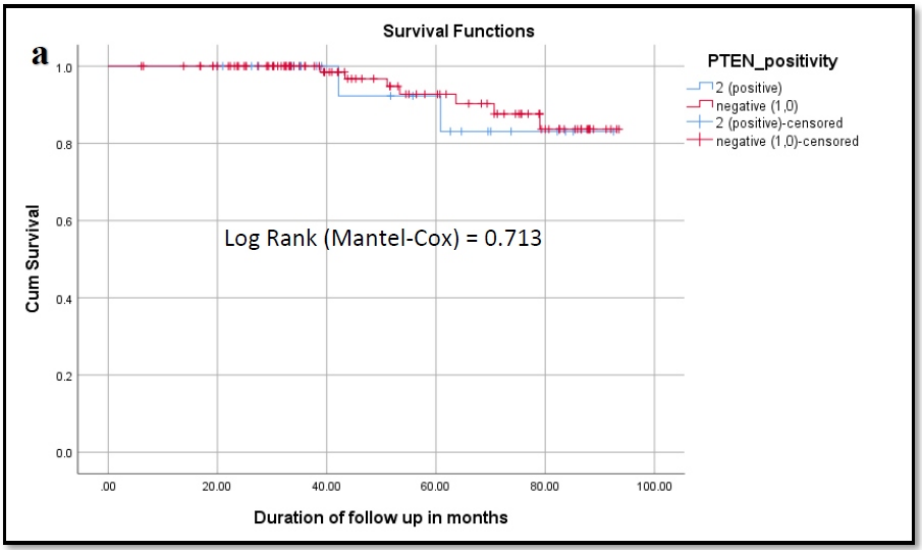

Figure 3: **a.** Comparison of overall survival in PTEN negative and positive groups

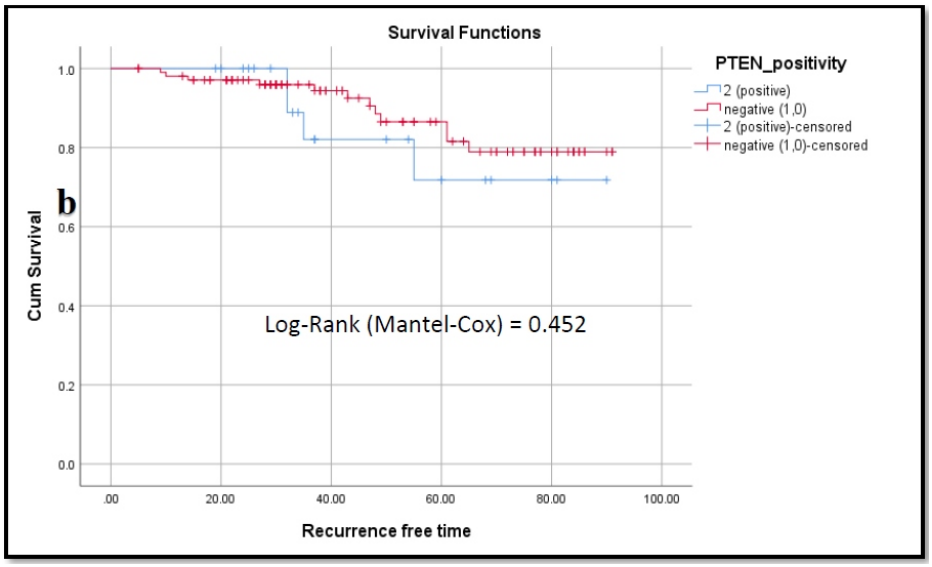

Figure 3: b. Comparison of Recurrence free survival in PTEN negative and positive groups.

Discussion

The aim of the study was to investigate the role of PTEN gene as a prognostic marker in breast cancer patients by analysing immunohistochemical expression of PTEN and analysing its association with recurrence of disease, stage, grade, tumour size and hormonal receptor status and to find out the association of PTEN with the survival in ER positive breast carcinomas treated with tamoxifen.

Only two studies, in the past have evaluated the PTEN expression in breast carcinoma with the tamoxifen resistance and they have demonstrated a poor survival in breast cancer patients with PTEN mutation compared to non-mutated ones (13, 14). Both the studies included small numbers of patients, 49 and 100. Both used immuno-histochemistry to evaluate the PTEN status, out of

those, one study had used genomic studies (fragment analysis) to evaluate the PTEN gene (14). Recurrence rate for breast cancer in both the studies were significantly high (57% and 47.9%). Survival studies must have a sufficient follow up to capture enough events and thereby ensure there is sufficient power to perform statistical tests. Although the number of participants is small in these two studies, because of longer follow up periods the comparatively higher number of events may have given a sufficient statistical power to the study.

Our study, which included 130 patients, did not demonstrate a relationship with PTEN expression and overall survival or recurrence free survival. However, the study showed that PTEN expression positively correlates with the level of ER expression (high and low). This means that PTEN negative patients are most likely to have low ER expression. Studies have proven that patients with high ER expression respond well to endocrine therapy compared to low expressers (15). In addition, our study demonstrated that PTEN expression negatively correlates with NPI value (patients with >5.4 and $\leq$5.4) and pathological stage of the tumour (patients in stage 1,2 and 3). It is well known that breast cancer prognosis is poor with tumours having NPI scores >5.4 as well as stage 3 tumours, compared to tumours in stage 1 and 2. Therefore, this study gives evidence favouring that loss of PTEN is a prognostic feature which signifies poor prognosis among breast cancer patients. Moreover, our study further gives evidence to support that PTEN can be lost even in patients with well-known good prognostic feature; ER positivity.

One of the above studies (13) also demonstrated a positive association of PTEN with ER expression and a negative association with LN metastasis and tumour recurrence but none of those studies showed a correlation of NPI and pathological stage with the PTEN expression.

Recurrence rate in our study group was very low (12.1%) compared to the above studies (57% and 47.9%) which may be the main reason why our study does not demonstrate a relationship with PTEN expression and survival. The number of events in the current study cohort appears not sufficient to substantiate an existing relationship. It can also be related to the mean follow-up period, which was nearly 4 years (51 months). In the other two studies the mean follow-up period was 72 (6 years) and 114 months (10 years) respectively (13, 14). The follow up period in our study is unlikely to be influencing the relationship between PTEN expression and survival because the study group which followed up to 6 years also demonstrated a significant association with loss of PTEN expression and survival, which was only around 2 years longer than our study. The discrepancy in the recurrence rate could be due to the fact that breast cancer patients are being managed well in the Sri Lankan health care system compared to the other two countries, Canada and Serbia, in the above study groups. This is further explained by the fact that in Sri Lanka, five year breast cancer survival is around 78.8%, which is not low compared to the USA figures (90%).

In this study the percentage of PTEN mutation (Low or absent expression) was 82.3%, which was higher than previously reported values (57% and 44.9%). The observed discrepancy can be related to the study population, which was a different population in a different part of the world as previous two studies were done in European countries, which has a different genetic composition. Other important reason for this discrepancy can be due to the sensitivity of the immunohistochemical analysis. In our thorough literature review, standardized reliable and reproducible methods for measuring PTEN expression on formalin-fixed tissue was lacking. The only study that was found was by Andrade *et al.,* who had developed a protocol for assessing PTEN status in formalin fixed breast cancer sample immunohistochemically. Our study was the first time that immunohistochemistry was used to evaluate PTEN status in tissues in Sri Lanka. Before commencing the study we have optimised and validated the immunohistochemistry method for PTEN.

In conclusion, though this study did not demonstrate a relationship between reduced PTEN protein expression with recurrence free survival and overall survival in tamoxifen treated patients, it was able to give further evidence that PTEN expression can be used as a prognostic marker in ER positive breast cancer patients as it showed a positive association with the level of ER expression

and negative association with NPI score and the pathological stage of the tumour. Further, this study also confirmed that a fraction (four fifths) of breast cancer patients that are categorized to have a better prognosis (ER Positivity) can have a poor prognostic feature; loss or poor PTEN expression. We would recommend further studies recruiting a larger study sample with a longer follow up to find out the association of PTEN with the survival in ER positive breast carcinomas in order to find out any relationship between loss of PTEN and tamoxifen resistance.

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