Original Article

The Effect of PT Link and Waterbath Antigenic Retrieval Procedures On The Expression of Common Receptors In Breast Cancer Cases

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ABSTRACT

Background: Antigen retrieval (AR) techniques play a major role in determining the quality and functional state of tissue proteins recovered from formalin fixed paraffin embedded blocks (FFPEB). Different techniques are used each with its merits and drawbacks.

Materials and Method: FFPEB from cases diagnosed as breast cancer were examined for the expression of estrogen- receptor (ER), progesterone receptor (PR) and HER2 by immunohistochemical techniques using two different AR methods.

Results: Fifty cases, 49 were from females, the age ranged from 28–85 years were examined. The expression of ER using water bath and PT link (pretreatment system) as antigen retrieval methods was equal, both methods showed 20(40%) positive cases and 30(60%) negative cases, whereas, the expression of PR was found positive in 18 cases (36%) and negative in 32 (64%) when water bath was used, in comparison to 10 positive (20%) and 40 negative (80%) when PT link was used (P value 0.312). The expression of HER2 was as follows; water bath HER2 weak positive 20 cases (40%), moderate expression 10 cases (20%), overexpression 2 cases (4%). PT link weak expression 13 cases (26%), moderate expression 15 cases (30%), overexpression 4cases (8%); yet, the total number of negative cases and positive cases was equal for both methods (p-value 0.035).

Conclusion: Antigen retrieval methods were found to affect the expression of common receptors in breast cancer. The magnitude of this effect was found to be significant in PR and HER2 receptors, however, the different methods of antigen retrieval did not affect the expression of ER. The appropriate and suitable retrieval methods have to be chosen individually for each receptor.

Keywords: Antigen retrieval, ER, PR, HER2 expression, PT link, waterbath.

Breast cancer is an ideal paradigm to study hormone-dependent tumors. Immunohistochemical testing revealed that around 70% of breast cancer cells are positive for estrogen (ER) and/or progesterone (PR) receptors. Stepping of breast lesions towards cancer is mainly influenced by estrogen. Thus, targeting ER using SERMs (selective estrogen-receptor modulators) is an effective therapeutic regimen for all stages of this disease[1]. As the most effective SERM, tamoxifen has been used as a major adjuvant treatment for primary breast cancer. But, efficiency wise over 50% of ER-positive tumors that initially respond to tamoxifen therapy will eventually develop resistance, resulting in recurrence and stepping forward of the cancer and the subsequent death of patients[1,2].

Recently molecular classifications of breast cancer draw much attention[3,4,5,6]. However, guidance of targeted therapy as tamoxifen and trastuzumab on immunohistochemical basis for tumors positive for estrogen / progesterone (ER/PR) receptors and Her2 (human epidermal growth factor receptor 2 is not far little specific compared to genetic and

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molecular-based guidance. Estrogen receptors are a group of proteins found inside cells. They are receptors that are activated by the hormone estrogen (17β-estradiol)[7]. Two classes of estrogen receptors exist ER, which is a member of the nuclear hormone family of intracellular receptors, and GPER, which is a member of the rhodopsin-like family of G protein-coupled receptors. This article refers to the former (ER)[8].

While ERα is obviously associated with more differentiated tumors, the involvement of ERβ is questionable. Different versions of the ESR1 gene have been identified (with single-nucleotide polymorphisms) and are associated with different risks of developing breast cancer[9].

Progesterone is a product of a single gene located on chromosome 11q22. Whereas, Progesterone receptor PR (NR3C3) is a protein existed inside cells[10]. Progesterone has 2 variants A and B having different molecular weights[11]. Binding of hormone to receptor prompted changes that cancel the inhibitory effect due to lack of hormone. These changes are prevented by progesterone antagonists.

Human Epidermal Growth Factor Receptor 2(HER2) also known as Neu, ErbB-2, CD340 (cluster of differentiation 340) or p185 is a protein that in humans is encoded by the ERBB2 gene. HER2 is a member of the epidermal growth factor receptor (EGFR/ErbB) family. Amplification or over-expression of this gene has been shown to play an important role in the pathogenesis and progression of certain aggressive types of breast cancer and in recent years it has evolved to become an important biomarker and target of therapy for approximately 30% of breast cancer patients. ERBB2 gene is overexpressed in nearly 30% of breast cancers and accompanied with increased disease recurrence and a poor prognosis[12].

Antigen retrieval is applied to unmask proteins as an initial step prior to immunohistochemical staining of formalin fixed paraffin embedded blocks[13]. In fact, different heating methods are in use for this purpose. This raised a lot of queries regarding how much and how heating methods are applied and their possible effects on staining intensity. Antigen retrieval affects the expression of ER / PR,HER2 receptor so it will finally affect the categorization of the disease and treatment policy.

This study aimed at the evaluation of the effect of antigen retrieval methods on the expression of ER and PR and HER2 receptors in patient suffering from breast cancer.

MATERIALS AND METHODS:
A retrospective descriptive cross-sectional study was conducted at Histopathology Department, University of Medical Sciences and Technology.

Fifty formalin-fixed, paraffin-embedded blocks (FFPE) were collected from histology laboratory at Ribat University Hospital, Alzuitona Hospital, and Total Lab Care Center. All blocks were from cases diagnosed as breast cancer. Sections of 5 micron thickness were obtained using rotary microtome. From each paraffin embedded block 6 sections were taken, in 3 sections antigen retrieval was done by PTlink antigenic retrieval and other 3 sections antigen retrieval was done by waterpath method. Each section has been stained for ER and PR and HER2/neu .

Tests for immunohistochemistry were performed using the procedure provided by Dako with kits, temperature-controlled waterbath, Dako Autostainer Slide Rack (Code S3704), Incubation container and cover, Retrieval solution, Tris-Buffered Saline (Code S3001) were used.

PTLink device automatically retrieves antigens in a way similar to the manually

set waterbath antigen retrieval, which incorporate preheat temperature, antigen retrieval temperature, and time as well as cool down settings. Typically, antigen retrieval is performed for 50 minutes at 97°C. Dako PTLink (Code PT100/PT101). Slides were reviewed by an experienced pathologist. Nuclear staining in more than 10% of tumor cells was considered positive for ER and PR. HER2 neu scoring was done according to ASCO/CAP guidelines (College of American Pathologists/American Society of Clinical Oncology)as follows: strong circumferential membranous staining in > 30% of invasive carcinoma cells = 3+; moderate, circumferential membranous staining in ≥ 10% of invasive tumor cells or 3+ in ≤ 30% of cells = 2+; weak and incomplete membranous staining in invasive tumor cells= 1+, no staining= 0. Tumors with 0 and 1+ staining were considered negative and cases scored as 2+ equivocal, and 3+ were considered positive, evaluated on 4× and 10× magnifications.

Data Analysis:
Data was analyzed by calculating the means and frequencies of receptors expression. Chi-square and P value were calculated to determine differences if any in receptors expression according to AR method. Data was analyzed by SPSS version16.

RESULTS:
Fifty patients were enrolled in the study, 1male and 49 females, with age range of 28–85 years. In this study the expression of estrogen-ER receptor using water bath and PT link as antigen retrieval methods was equal with 20(40%) positive cases and 30(60%) cases were negative, methods of antigen retrieval did not affect the expression of ER. Whereas, the expression of PR receptor was found 18(36%)

Table (1): The distribution of study group according to the type of cancer.

Type of carcinoma	Frequency	Percent
Invasive ductal carcinoma	44	88.0
Lobuler carcinoma	5	10.0
Phylloides	1	2.0
Total	50	100.0

positive and 32 negative cases (64%) when water bath was used, and 10 positive (20%),40 negative(80%)when PT link was used. Therefore, there was a difference in the expression of PR by using water bath and PT link, this difference was not statistically significant (P value 0.312).The expression of HER2 was as follows: water bath HER2 weak positive 20 cases (40%), moderate expression 10 cases (20%), overexpression 2 cases (4%). PT link weak expression 13 cases (26%), moderate expression 15 cases (30%), over expression 4cases (8%). The total number of negative cases and positive cases was equal. The difference was statistically significant (p-value 0.035).
Positivity and negativity of HER2 was not affected by the method of antigen retrieval. But, the degree of expression of HER 2 was significantly affected by the different methods of antigen retrieval.PT link showed better expression of HER2.

DISCUSSION:
Antigen Retrieval has improved the diagnostic accuracy through reducing the false negative results and also making results more reproducible. So expression of receptors and treatment protocols will be affected very much, i.e. the use of anti-hormonal drugs like tamoxifen and herceptin.
In this study, the expression of ER was found to be equal by using waterbath and PT link and ER-positive 40% and ER-negative was found to be 60%.

Table (2): The expression of estrogen receptors (ER) and progesterone receptors (PR) using waterbath and PT link as antigen retrieval methods.

Retrieval method	ER positive	ER negative		PR positive	PR negative		total
Waterbath	20	30		18	32		50
PT link	20	30	P value=0.018	10	40	P value=0.312	50
total	40	60		28	72		100

Table (3): The expression of HER-2 using water bath and PT link as antigen retrieval methods

| Retrieval Method | HER-2Expression | | | | Total | P value |
	HER-2Positive+	HER-2Positive++	HER-2Positive+++	HER-2 Negative		
Waterbath	20	10	2	18	50	0.035
PT link	13	15	4	18	50	
Total	33	25	6	36	100	

These figures were in agreement with (Elgaili *et al*. 2010)[14] who found ER negativity 68.3% and the findings are different from study in Nigeria that estimated ER-negative cases by 72% of the cases which was higher than the figures recorded in Sudan.

In the present study, the expression of progesterone was found to be higher when waterbath was used as antigen retrieval method compared to PT link, 36% versus 20%, so PR negativity would be 64% and 80%. In the study done by Elgaili *et al*. progesterone negative was found to be 69.5%[14].This percentage is in agreement with that scored by using waterbath as compared to negativity of 80% found with the use of PT link. Therefore, waterbath improved antigen expression when compared to PT link regarding progesterone receptors. Waterbath results were similar to previous studies. That is to say, waterbath is more superior for antigen retrieval when compared to PTlink for progesterone receptors.

In our study, the expression of HER2 as positive and negative was equal, and negativity HER2 was found to be 36%.

In the present study the expression of HER2 was found 20 cases weak expression,10 cases moderate and 2 cases overexpression by using PT link, and were found 13 cases weak,15 cases moderate and 4 cases overexpression by using PT link, The difference in expression was found to be statistically significant. This finding indicates that PT link use improves the expression of HER2 in comparison to waterbath .

One study compared the staining intensity of HER2 by using waterbath and microwave as methods of antigen retrieval and it was documented that staining intensity was found to be better by using waterbath than microwave.

Since both PT link and waterbath are heat-induced epitope retrieval methods, other methods should be assessed separately. Standardization of fixation as well as antigen retrieval methods for each antigen to attain best result in IHC.

CONCLUSION:

Waterbath and PTLink methods showed similar results regarding the expression of ER Estrogen receptors. There was a difference in expression of PR progesterone receptors by changing method of retrieval. Waterbath demonstrated better expression of PR progesterone compared to PT link.

Positivity and negativity of HER2 was not affected by the method of antigen retrieval. But, the intensity of expression of HER 2wassignificantly affected by methods of antigen retrieval.PT link showed better expression of HER2.

CONFLICT OF INTEREST:
None of the authors has any conflict of interest to disclose

ETHICAL CLEARANCE:
This work was approved by the ethical committee at University of Medical Sciences and Technology, Faculty of Medical Laboratory Sciences.

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