Original article:

Expression of B-cell lymphoma-2, Vimentin and CD44V6 in normal tissue around breast ductal carcinoma in sample of Iraqi women

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Abstract:
Background: Breast cancer is the most common neoplasm among women in Iraq and in developed countries. The aim of this study was to study the immunohistochemical reaction pattern of epithelial, myoepithelial cells in normal tissue around breast carcinoma in addition to determine the sensitivity, specificity and validity of predetermined cut-off score of Bcl2, vimentin, CD44v6 markers and to determine the prognostics significances of these markers in future conservative breast surgery.

Materials and Methods: A total of 52 samples from breast tissue were included in this study. There were 12 histological samples of normal breast tissue as a control group and 40 samples contain (28) samples of normal tissue around infiltrative ductal carcinoma and (12) samples of normal tissue around ductal carcinoma in situ. For each case, 4 sections of 4 µm thickness were taken; one representative section was stained with H&E stain and other three sections stained immunohistochemically for each marker.

Results: there was significant increase in Bcl2 staining in normal tissue around DIS compared to control group and infiltrative DCA (P = 0.001 and 0.004 respectively). Also there was significant increase in Vimentin staining in normal tissue around DCIS (91.7%) compared to control group (P = 0.000), together with normal tissue around infiltrative DCA (82.1%) of the cases compared to control group, CD44v6 shows greater extent of staining in normal tissue around DIS compared to control group and infiltrative DCA (P 0.001, 0.044 respectively).

Conclusion: There was higher expression of Bcl2, Vimentin and CD44V6 in normal tissue around breast carcinoma in comparison to normal breast tissue.

Keywords: breast cancer, vimentin, CD44V6, BCI2, normal tissue around breast CA

Background:
The breasts are specialized accessory glands of the skin that secrete milk [Richard, 2012]. They are largely superficial and anterior to the thoracic wall [Moore & Dalley, 2008]. The human breast is a dynamic organ that undergoes different developmental stages through women life and does not reach full maturity unless a woman experiences pregnancy and childbirth [Donna, 2007]. In non-pregnant female the parenchymal structure of the gland consists from many lobes, each lobe consists of many lobules with a structural unit called terminal duct lobular unit (TDLU) [Anthony, 2012]. Each lobule has several branching ducts, but the secretory units are still small and rudimentary. Stratified
cuboidal epithelium lined the lactiferous sinuses and simple cuboidal epithelium lined the lactiferous ducts and terminal ducts that covered by closely packed myoepithelial cells. Sparse fibers of smooth muscle also encircle the larger ducts [William, 2009]

**Materials and methods:**
*Clinical data:* a total of 52 tissue biopsies from breast tissue were included in this retro and prospective study. There were 12 histological samples of normal breast tissues as a control group and 40 samples contain normal tissue surrounded the cancerous tissue and as the following:

**Normal tissue around infiltrative ductal carcinoma are** (28) samples.

**Normal tissue around ductal carcinoma in situ are** (12) samples.

The prospective tissue samples were (28) and they were collected from Al- Yarmook teaching hospital, Al-khadimiya teaching hospital, Baghdad teaching hospital and two private laboratories, for the period from October 2013 to June 2014.

The retrospective tissue samples were (24) obtained from archival paraffin embedded blocks selected for the period from October 2012 to October 2013 from histopathological files of Al-Yarmook teaching hospital.

These patients either had modified mastectomy (29) or excisional biopsy (11). The clinicopathological parameters were obtained from available histological reports.

The remaining 12 samples which comprise a normal control group were obtained from female who undergo reduction mammoplasty, these samples were taken from female’s breast their mean age was 48 years with a range of 30-76 years.

Ethical approval for use of the specimen was obtained and histopathological diagnosis was confirmed by review of freshly prepared hematoxylin and eosin stained slides.

The patient’s age ranged from 30 to 76, with mean age of 45.5. They were divided according to their age into three levels (Table 2).

**Table 1:** Showed type and number of tissue in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Types</th>
<th>No. of patients</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control</td>
<td>12</td>
<td>23.076 %</td>
</tr>
<tr>
<td>II</td>
<td>Normal tissue around Invasive cancer (NTAICA)</td>
<td>28</td>
<td>53.846 %</td>
</tr>
<tr>
<td>III</td>
<td>Normal tissue around in situ Carcinoma (NTAISCA)</td>
<td>12</td>
<td>23.076 %</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>52</td>
<td>100 %</td>
</tr>
</tbody>
</table>
Table 2: Showed age levels classification and number of cases in each level.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Normal</th>
<th>NTAIC</th>
<th>NTAISC</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 40</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>11(21.15%)</td>
</tr>
<tr>
<td>40-49</td>
<td>4</td>
<td>14</td>
<td>4</td>
<td>22(42.30%)</td>
</tr>
<tr>
<td>=&gt;50</td>
<td>4</td>
<td>12</td>
<td>3</td>
<td>19(36.53%)</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>28</td>
<td>12</td>
<td>52(100%)</td>
</tr>
</tbody>
</table>

Tissue preparation

The breast tissues were histologically prepared for paraffin section according to [Bancroft and Stevens, 1987][12] as follows: Fixation, dehydration, clearing, impregnation, embedding, sectioning, dewaxing, hydration, staining and mounting. For each case, 2 serial sections of 4 µm in thickness were taken; one representative section was stained with H&E stain according to standardized criteria and the second one was stained with immunohistochemical marker.

Antibodies

Table 3: primary antibody kits

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>source</th>
<th>type</th>
<th>dilution</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD44v6 antibody</td>
<td>Abcam Clone[VFF-7] code:ab30436</td>
<td>Mouse antihuman monoclonal</td>
<td>1:100</td>
<td>Normal breast tissue</td>
</tr>
<tr>
<td>Anti-Bcl-2 antibody</td>
<td>Abcam Clone:[Bcl2/100] Code:ab117115</td>
<td>Mouse antihuman monoclonal</td>
<td>1:100</td>
<td>Normal breast tissue</td>
</tr>
<tr>
<td>Anti-vimentin antibody</td>
<td>Abcam Clone:[RV202]</td>
<td>Mouse antihuman monoclonal</td>
<td>1:100</td>
<td>Normal breast tissue</td>
</tr>
</tbody>
</table>

Table 4: secondary detection kits

<table>
<thead>
<tr>
<th>Materials</th>
<th>Quantity</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Peroxidaes block</td>
<td>15 ml</td>
<td>3% hydrogen peroxide water biotin labeled affinity isolated</td>
</tr>
<tr>
<td>2- Biotinylated Goat anti-mouse</td>
<td>15 ml</td>
<td>Goat anti-mouse immunoglobulins in PBS containing stabilizing protein and 0.01 mol/L sodium azide.</td>
</tr>
<tr>
<td>3- Streptavidin-HRP</td>
<td>15 ml</td>
<td>Streptavidin conjugated to horse radish peroxidase in PBS containing stabilizing protein and antimicrobial agents.</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------</td>
<td>---------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>4- Substrate: DAB substrate buffer</td>
<td>15 ml</td>
<td>Imidazole HCL buffer pH 7.5 containing hydrogen peroxide and antimicrobial agent.</td>
</tr>
<tr>
<td>5- DAB chromogen</td>
<td>1 ml</td>
<td>3-3 diaminobenzidine in chromogen solution</td>
</tr>
</tbody>
</table>

### Assessment

All tissues were assessed without knowledge as to whether it was from a cancer case or a control, and of the age of the patient.

#### Semiquantitative Immunohistochemical Scoring [IHS]:

For assessment, (6) normal lobules were identified for each sample and graded according to the extent and intensity of staining. The positive control for each batch of staining was used as the reference point for assessing intensity. Intensity was scored:

- 0 (no staining),
- 1 (weak),
- 2 (moderate) and
- 3 (strong).

Extent of staining was categorized by proportion:

- 0–25% (0.25) of cell stained positively
- 26–50% (0.50)
- 51–75% (0.75)
- 76–100% (1.00)

An index for each lobule, between 0 and 3, was generated by multiplying these two scores, and the mean of the 6 lobules gave the index for that tissue [13].

Where all tissues had been analyzed by light microscopy (Micros Austria ® microscope with a LCD touch screen). The data were linked to clinical information for statistical analysis.

#### Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22).

Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values).

The significance of difference of different means (quantitative data) were tested using Students-t-test for difference between two independent means or Paired-t-test for difference of paired observations (or two dependent means), or ANOVA test for difference among more than two independent means. The significance of difference of different percentages (qualitative data) was tested using Pearson Chi-square test ($\chi^2$-test) with application of Yate's correction or Fisher Exact test whenever applicable.
Results:

The extent of IHC staining of Bcl2 marker:

Group III was showed significant increase in the extent and total scoring of Bcl2 staining compared to both Group I and Group II ($P = 0.001$).

Group III was showed significant increase in the extent of Bcl2 staining and total scoring compared to Group II ($P$ value $= 0.004$).
The extent of IHC staining of vimentin marker

**Figure 4:** Histogram of the extent of vimentin staining in each group.

**Figure 5:** Box plot showed the IHS (index) of vimentin in each group.

- There were highly statistical Significant difference in vimentin staining and scoring among the three groups (p value = 0.000)

- There were no Significant difference in vimentin staining and scoring between the group II and group III (p value = 0.560)

The extent of IHC staining of CD44v6 marker

- Group III shows greater extent of CD44v6 staining and total scoring than both Group I and Group II (p value = 0.001)

- Group III shows greater extent of CD44v6 staining and total scoring than Group II (p value = 0.044)
Figure 7: Box plot showed the IHS (index) of CD44v6 in each

Figure 8: Microphotograph of IHC study of BCL2 marker in normal breast tissue show negative reactivity to BCL2 in epithelial cells of mammary duct. X100

Figure 9: Microphotograph of IHC study of BCL2 marker in normal breast tissue around infiltrative CA show positive (+) reactivity with mild intensity to Bcl2 in epithelial cells of mammary duct (red arrows). X400
**Figure 10:** Microphotograph of IHC study of BCL2 marker in normal breast tissue around infiltrative CA show positive (+++) reactivity with strong intensity to BCL2 in epithelial cells of mammary duct (red arrows). X400

**Figure 11:** Microphotograph of IHC study of BCL2 marker in normal breast tissue around infiltrative CA show positive (+++) reactivity with strong intensity to BCL2 in epithelial cells of mammary duct (red arrows). X400
Discussion

Expression of IHC staining of B-cell lymphoma 2 in all groups:

This study reveal that: 75% of normal breast tissue and 67% of normal tissue around infiltrative ductal carcinoma had shown negative reactivity against Bcl2 antibody while there is only 8.3% of normal tissue around ductal carcinoma in situ had showed negative reactivity to Bcl2 antibody. while there were 91.7% of total cases of NTAISCA had mean of IHS (1.18±0.86) and 33% of NTAICA had mean of final IHS (0.51±0.69) and only 25% of normal breast tissue had mean of IHS (0.28±0.27), those had shown positive reactivity against Bcl2 antibody. These results agree with previous studies like[Hassan H I et al 1998, Adams J M et al 2007, Batchelder A. J. et al 2009] [14][15][13] they all demonstrated there is a significantly higher level of expression of anti-apoptotic protein (Bcl2) in the normal tissue around breast carcinoma in comparison to age-matched breast tissue from women without cancer.

Also there was high expression of Bcl2 in normal tissue around in situ ductal carcinoma (91.7%) comparing to the normal tissue around infiltrative carcinoma(33%) which is agreed with previous studies of [16] who work on breast carcinoma and state; Bcl2 expression was more in the patients with ductal carcinoma in situ than patients who had infiltrative carcinoma. This is because Bcl2 expression was lost when there is progression in breast cancer as noted by [17][18]. This affirms the loss of bcl-2 expression, and when it occurs, it is a relatively late event in the progression of the disease.
These also agreed with [Sierra A et al., 1996; Rostamizadeh L, 2013][19][20], they work on cancerous tissue; state that Bcl-2 protein expression was seen in the majority of the infiltrative carcinomas; its incidence varying according to tumor grade where they found a very close and statistically significant direct relationship between Bcl-2 positivity with low grade cancer and/or well-differentiated tumours.

The results for NTAISCA agree with previous studies like [13][21]. They demonstrate the differences in expression of BCL2 which present in all age levels, but it is greater in age group of more than 50 years.

Also agreed with studies on cancerous tissue like [23][24][25][7]. They noted; there were significant differences between cases of premenopausal which had frequently negative results in comparison with the positive results of postmenopausal state.

**Expression of IHC staining of vimentin in all groups:**

This study reveals that 91.7% of normal tissue around ductal carcinoma in situ had mean of IHS (1.65±1.07) and 82.1% of cases of normal tissue around infiltrative carcinoma had mean of IHS (1.25±0.94), had shown positive reactivity against Vimentin antibody while there are 17.9% of cases of NTAICA and 8.3% of cases of NTAISCA had shown negative vimentin staining comparing to 100% of cases of normal control group that had negative vimentin staining. Also there was no statically significant difference in vimentin staining in NTAISCA and NTAICA regarding the same age groups.

There is no statistical significant difference between normal tissue around in situ CA and normal tissue around infiltrative CA in expression of Vimentin staining. This is in concordance with previous studies on cancerous tissue like [Heatley M et al., 1993; Kaya H et al., 2008] which state that there were positive vimentin expression in both carcinoma in situ and infiltrative ductal carcinoma.[David J et al., 2006] who work on cancerous breast tissue state; 94% of cases were positive for vimentin staining.

Our most significant and previously unreported finding; there was significant increase in vimentin staining in normal tissue around infiltrative ductal carcinoma in relation to all age levels when compared to the normal control group. We found that there was no significant inverse correlation (-0.209) between vimentin marker and age, where p value was 0.286. In spite of Nuha M et al., 2014 state that there was vimentin staining in all cases of infiltrative ductal carcinoma and in all age groups. Our most significant and previously unreported finding; there was significant increase in vimentin staining in normal tissue around infiltrative ductal carcinoma in relation to all age levels when compared to the normal control group. We found that there was no significant inverse correlation (-0.209) between vimentin marker and age, where p value was 0.286. In spite of Nuha M et al., 2014 state that there was vimentin staining in all cases of infiltrative ductal carcinoma and in all age groups.

**4.2.3 Expression of IHC staining of CD44v6 in all groups:**

The results of this study reveal that 91.7% of cases of normal tissue around in situ ductal carcinoma had mean of IHS (1.53±0.97) and 53.6% of cases of normal tissue around
infiltrative ductal carcinoma had mean of IHS (0.64±0.66) both had shown positive reactivity against CD44v6 antibody compared to 8.3% of cases of NTAISCA and 46.4% of cases of NTICA had shown negative reaction to CD44v6 antibody, also there were no cases in normal control group had shown positive reaction to CD44v6 antibody, so the normal tissue around in situ CA show greater extent of expression of CD44v6 staining than normal control group and normal tissue around infiltrative CA.

This data was coincide with previous studies which worked on cancerous tissue like [28], they reported that the positive expression rate of CD44v6 is 80% in primary tumor and 100% in focal tumor, but no expression is found in normal mammary tissue. [29] Stated that there were 94% of cases stained with CD44v6 with moderate to strong intensity. [30] Demonstrated higher and lower expression of CD44v6 in breast carcinoma. [32][31] suggested the expression of CD44v6 had determined by differentiation status of the tumor cells where [33] observed progressive loss of CD44v6 as tumor become less differentiated. This may be speculated that the absence of variant isoforms at the cell surface may facilitate cell detachment and favor interaction of CD44 standard with the extracellular matrix to permit migration and invasion. So Loss of CD44v6 facilitated matrix and vascular infiltration and dissemination of breast carcinoma cells. As was shown by [34 and 35] who were reported that there were no cases stained for CD44v6 in normal breast tissue.

Where this study reveal that there was strong significance inverse correlation (-0.726) between age and CD44v6 marker in normal control group where p value was 0.008. While there was no significant inverse correlation (-0.253) between age and CD44v6 marker in NTAICA where p value was 0.195 and there was significant weak inverse correlation between age and CD44v6 marker in NTAISCA where p value was 0.183. These results coincide with [36 and35] which had been shown expression of CD44v6 in intraductal carcinoma, and was related with tumor invasion and metastasis, but not with the age of patients. This study reveals that there was statistical significant difference between CD44v6 IHS means in normal tissue around infiltrative CA compared to mean of normal control group in age groups of less than 40 and more than 50 years, also there was statistical significant difference between CD44v6 IHS means in normal tissue around in situ CA compared to normal control group in age groups of less than 40 years and 40-50 years and there was statistical significant difference between CD44v6 IHS means in normal tissue around in situ CA compared to normal tissue around infiltrative CA in age group of 40-50 years; so our most significant and previously unreported finding that there was increasing in CD44v6 expression in age group of 40-50 years especially in normal tissue around in situ carcinoma.

Where this study reveal that there was strong significance inverse correlation (-0.726) between age and CD44v6 marker in normal control group where p value was 0.008. While there was no significant inverse correlation (-0.253) between age and CD44v6 marker in NTAICA where p value was 0.195 and there was significant weak inverse correlation between age and CD44v6
marker in NTAISCA where p value was 0.183. These results coincide with [36][35]

**Conclusions:**

This study concludes that:

1. There was direct correlation in expression of anti-apoptotic protein (Bcl2) with age especially in normal tissue around ductal carcinoma in situ; while there was only increased expression of Bcl2 in age group of less than 40 years in normal tissue around infiltrative ductal carcinoma.

2. Expression of vimentin staining was in all age levels.

3. This study couldn’t find strong inverse correlation between expression of CD44v6 and age levels.

4. This study show direct correlation between Bcl2 with CD44v6 in normal control group and between CD44v6 and vimentin in normal control group and NTAICA and between Bcl2 and vimentin in NTAICA and couldn’t find any correlation between markers in NTAISCA.

5. For testing prognosis of normal tissue around breast carcinoma, vimentin had more sensitivity and specificity and its cut-off score are more valid than other markers in NTAICA and NTAISCA. Then CD44v6 came after vimentin in sensitivity and specificity and validation of its cut-off score then Bcl2 came after that can used only in NTAISCA. The sensitivity of these markers is greater in normal tissue around ductal carcinoma in situ.

**Recommendations**

1. Further studies with large number of NTAICA and NTAISCA cases using more additional more specific antibody like KI-67 and p53 with Bcl2 and cytokeratin markers with vimentin and c-cahdrin with CD44v6.

2. Further studies with large number of cases of NTAICA and NTAISCA using ER, PR receptor markers to identify the correlation between our markers and menopausal status.

3. Further researches on normal breast tissue around different type of carcinoma.

4. Further researches about using of our markers in trucut biopsy for conservative surgery.

5. Using of our markers on fresh frozen section instead of paraffin embedded blocks because in this study there was difficulty in antibody retrieval of old blocks also to explain the difference in using both approach.

6. Further study about using of our markers in advanced pathological technique (ISH, DNA microarray), to identify the gens that responsible for appearance of anti-apoptotic protein (Bcl2), mesenchymal marker (vimentin), and adhesion molecule (CD44v6) and identify its location.
References


