Differentiation of RAGE Expression in Primary Tumors and Lymph node Metastatic Deposits of Breast Carcinoma

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Abstract: Breast cancer is a complex disease that results from the inheritance of a number of susceptible genes. Intensive search work was conducted worldwide on molecular bases of breast cancer in order to achieve the best therapeutic modalities; however, breast cancer still remains a challengeable task. The selection of RAGE gene as a research candidate is biologically plausible. RAGE is a member of the immunoglobulin superfamily of cell surface receptors, and its interaction with advanced glycation end products and other molecules plays a role in the pathogenesis of cancer progression and metastasis. Despite great strides in treatment for localized breast cancer, metastatic breast cancer remains an overwhelmingly lethal disease.

Previous studies showed that lymph node deposits of cancer breast showed more expression of RAGE than the primary tumor. In this study we evaluate RAGE expression in primary breast cancer and in lymph node metastases. We found that most of RAGE expression parameters were increased with progression of primary tumor grade and stage. We reported also a higher expression of RAGE parameters in metastatic axillary lymph node deposits compared to its primary breast cancer. These findings support the validity of anti-RAGE therapy for treatment of both primary and nodal metastatic breast cancer.

Keywords: Breast cancer, RAGE, immunohistochemistry

Introduction:

Breast cancer is the most common form of malignancy that has serious implications on the health of women worldwide, and ranks as the second leading cause of mortality in women. The main cause of breast cancer is still not completely understood [21]. Breast cancer may develop by different factors such as genetic alteration, lifestyle, and obesity, higher level of specific hormones, tobacco and smoking. For instance, several studies have suggested that chronic inflammation is derived from smoking and tobacco, which increases the risk of breast cancer [8, 13]. The pathology of breast cancer is classified into three categories according to its gene expression, receptors and immunohistochemistry characterization. This influences the prognosis of the disease and treatment response [5, 18].

Many risk factors increase the chances of developing breast cancer. Notably, age is considered the primary risk factor related to breast cancer. For instance, women aged under 25 are less likely to develop breast cancer as there are 10 cases reported per 100,000 women and at the age of 45. These cases rise by 100-fold [4]. The human diet consists of a combination of both carcinogenic and anti-carcinogenic compounds contained within various foods. Specific types of these compounds may lead to the production of free oxygen radicals that cause DNA damage [4]. The connection between obesity and breast cancer related to menopause where it is due to the high levels of endogenous oestrogen found in obese women who are postmenopausal. On the other hand, women who are premenopausal and obese are less likely to develop breast cancer [12]. An alternative known risk factor of breast cancer is mammography density, where it is irrelevant whether the women are in the pre- or postmenopausal stage [6]. The family history is another important risk factor, where a meta-analysis has previously demonstrated that there is a 12% chance of having another family member affected and 1% chance of having more than one family affected by breast cancer [1, 4].

RAGE is a member of the immunoglobulin superfamily of cell surface receptors, and its interaction with advanced glycation end products...
and other molecules plays a role in the pathogenesis of cancer progression and metastasis [9]. Converging evidence suggested that circulating RAGE level may be a novel clinical biomarker for many types of cancers, such as lung cancer, prostate cancer, colorectal cancer, breast cancer [22]. Experimental studies have demonstrated that to block RAGE signaling in mice can reduce the migration and invasively of tumor cells, and possibly cell proliferation and the production of tissue metalloproteinase [19]. Further studies that examined the genetic backgrounds of RAGE gene found that circulating RAGE level was largely determined by its genetic defects [2]. As RAGE genomic sequence is highly polymorphic, it is of interest to determine which genetic defects have a functional potential to affect the final bioavailability of RAGE, and thus the development of breast cancer [9]. RAGE expression is upregulated widely in aggressive triple-negative breast cancer (TNBC) cells, both in primary tumors and in lymph node metastases [14]. According to Nasser et al, (2015) in evaluating the functional contributions of RAGE in breast cancer, found that RAGE-deficient mice displayed a reduced propensity for breast tumor growth. In an established model of lung metastasis, systemic blockade by injection of a RAGE neutralizing antibody inhibited metastasis development [17]. Mechanistic investigations revealed that RAGE bound to the proinflammatory ligand S100A7 and mediated its ability to activate ERK, NF-kB, and cell migration. In an S100A7 transgenic mouse model of breast cancer (mS100a7a15 mice), administration of either RAGE neutralizing antibody or soluble RAGE was sufficient to inhibit tumor progression and metastasis [11]. Overall, in the current study, we aim to evaluate RAGE as a candidate biomarker for diagnosis and grading of breast cancer as well as to test the link of inflammation to aggressive breast cancer development and the possible role of RAGE as target for primary and metastatic breast cancer-therapy.

Material and Methods:

Our study was conducted on breast and lymph node biopsies from 58 female patients ranging in age from 18 to 55 years. Tissue samples included excision biopsies from benign cases and modified radical mastectomy biopsies from malignant cases. Benign lesions include fibrocystic disease of breast (6 cases) and fibroadenoma (2 cases), while malignant tumors include invasive duct carcinoma (47 cases), invasive lobular carcinoma (2 cases), medullary carcinoma (1 case) and intraduct carcinoma (2 cases).

Invasive duct carcinoma cases include 3 cases of tubular carcinoma, 2 cases of mucoid carcinoma and 40 cases of non-otherwise specified (NOS) carcinoma. Sections from these biopsies were subjected to the following procedures:

1) Routine histopathological examination using paraffin sections stained by hematoxylin and eosin stain, with special reference to:
   - Diagnosis benign and malignant lesions
   - Diagnosis of grade, stage and type of breast carcinoma
   - Diagnosis of metastatic deposits in regional lymph nodes

2) Immunohistochemical study of tissue sections using monoclonal antibody against RAGE protein.

Immunohistochemical Method:

Anti-RAGE antibody (Santa Cruz Biotechnology) was used for immunohistochemical (IHC) detection of the expression of RAGE protein in tissue. Tissue sections were processed for IHC analysis of RAGE protein as follows. IHC examinations were carried out on 3 μm thick sections. For anti-RAGE IHC, unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, at 90°C for 30 min. Sections were incubated in 0.03% hydrogen peroxide for 10 min at room temperature, to remove endogenous peroxidase activity, and then in blocking serum (0.04% bovine serum albumin, A2153, Sigma-Aldrich, Shanghai, China, and 0.5% normal goat serum X0907, Dako Corporation, Carpenteria, CA, USA, in PBS) for 30 min at room temperature. Anti-RAGE antibody (A11): sc- 80652 RAGE Antibody (A11) is a mouse monoclonal IgG2a provided at 200 μg/ml, raised against a truncated extracellular domain of RAGE of human origin (Santa Cruz Biotechnology, USA). The antibody was used at a dilution of 1:100. The antibody was incubated overnight at 4°C. Sections were then washed three times for 5 min in PBS. Non-specific staining was blocked 5% normal serum for 30 min at room temperature. Finally, staining was developed with diaminobenzidine substrate and sections were counterstained with hematoxylin. PBS replaced RAGE antibody in negative controls.

Quantification of protein expression:

The expression of RAGE was semi quantitatively estimated as the total membrane-cytoplasmic immunostaining scores, which were calculated as...
the product of a proportion score and an intensity score. The proportion and intensity of staining was evaluated independently. The proportion score reflected the fraction of positive staining cells (score 0: <5%, score 1: 5%-10%, score 2: 10%-50%, score 3: 50%-75%, score 4: >75%).

and the intensity score represented the staining intensity (score 0: no staining, score 1: weak positive, score 2: moderate positive, score 3: strong positive). Finally, a total expression score was given ranging from 0 to 12. Based on the analysis in advance, RAGE was regarded as negative expression in gastric cancer tissues if the score <2, and positive expression if the score ≥2 [3].

Statistical analysis
Pearson's Chi square test was used to compare the differences in percentages of positive results between groups. ANOVA and student t-tests were used to compare groups' means. SPSS 20.0 for Windows was used for all statistical analyses. Significant differences between groups were achieved if (p<0.05).

Results:
Our study was conducted on breast and lymph node biopsies from 66 female patients ranging in age from 18 to 55 years. The mean age for benign cases was 31.45±9.22 years and for malignant cases was 48.65±13.81 (p <0.01). Tissue samples include 6 excision biopsies from benign cases and 60 modified radical mastectomy biopsies from malignant cases.

Benign lesions include fibrocystic disease of breast (4 cases) and fibroadenoma (2 cases), while malignant tumors include invasive duct carcinoma (50 cases), invasive lobular carcinoma (2 cases), medullary carcinoma (1 case), mucoid carcinoma (1 case) and intraduct carcinoma (6 cases).

Invasive duct carcinoma cases include 3 cases of tubular carcinoma, and 47 cases of non-otherwise specified (NOS) carcinoma.

The grades and stages of malignant tumors were summarized in graph (1). Most of the studied cases were of low grade but high stage of malignancy (GI&II, T2&3).

Graph (1): Distribution of studied malignant cases according to (A) grade and (B) stage.

There were high significant differences in means of all RAGE expression parameters between benign and malignant breast lesions. Parameters of RAGE expression were also higher in lymph node metastatic deposits in relation to primary breast tumors. (Table 1).

Table (1): Difference in RAGE expression parameters between benign and malignant breast lesions, compared to lymph node (LN) metastasis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>RAGE %</th>
<th>RAGE intensity</th>
<th>RAGE score</th>
<th>LN RAGE %</th>
<th>RAGE Intensity</th>
<th>LN RAGE score</th>
</tr>
</thead>
<tbody>
<tr>
<td>benign</td>
<td>Mean</td>
<td>3.3333</td>
<td>1.0000</td>
<td>1.0000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>1.36626</td>
<td>0.0000</td>
<td>0.0000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>malignant</td>
<td>Mean</td>
<td>63.6154</td>
<td>2.0233</td>
<td>6.5769</td>
<td>86.6667</td>
<td>2.4167</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>29.13603</td>
<td>.74096</td>
<td>3.80065</td>
<td>.858830</td>
<td>.94822</td>
</tr>
<tr>
<td>Total</td>
<td>Mean</td>
<td>57.3793</td>
<td>1.8966</td>
<td>6.0000</td>
<td>86.6667</td>
<td>2.4167</td>
</tr>
</tbody>
</table>
Low grade invasive breast cancer show higher percentage of RAGE expression compared to DCIS and high grade invasive breast cancer with significant difference between groups (p<0.01).

RAGE intensity and RAGE score showed increasing values from DCIS up to high grade invasive breast carcinoma. However, the difference between groups was not statistically significant (p>0.05).

Intensity and score of RAGE expression were higher in lymph node metastatic deposits of high grade invasive breast cancer compared to the same parameters in case of low grade invasive breast cancer with statistical significance (p<0.05 and p<0.01 respectively). On the other hand the percentage of RAGE expression in lymph node of metastatic deposits was non-significantly higher in low grade invasive breast cancer compared to cases of high grade invasive breast cancer (p>0.05).

### Table (2): Difference in RAGE expression parameters in relation to different grades of malignancy and lymph node metastatic deposits:

<table>
<thead>
<tr>
<th>GRADE</th>
<th>RAGE %</th>
<th>RAGE intensity</th>
<th>RAGE score</th>
<th>LN RAGE %</th>
<th>LN RAGE intensity</th>
<th>LN RAGE score</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCIS N</td>
<td>Mean</td>
<td>4.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>1.41421</td>
<td>0.0000</td>
<td>0.0000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Low grade</td>
<td>Mean</td>
<td>66.9565</td>
<td>2.2457</td>
<td>6.957</td>
<td>86.8182</td>
<td>2.3636</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>27.02834</td>
<td>0.73030</td>
<td>3.6577</td>
<td>8.83284</td>
<td>9.2273</td>
</tr>
<tr>
<td>High grade</td>
<td>Mean</td>
<td>55.0000</td>
<td>2.5000</td>
<td>8.0000</td>
<td>85.0000</td>
<td>3.0000</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>28.86751</td>
<td>.57735</td>
<td>4.61880</td>
<td>5.77350</td>
<td>.00000</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td>P&lt;0.01</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

There is increase in the value of all RAGE expression parameters from DCIS up to high stage of invasive breast cancer, however, the difference was significant in case of RAGE percent (p<0.001) and non-significant in RAGE intensity and RAGE score parameters (p>0.1 and p>0.05 respectively). All parameters were higher in lymph node metastatic deposits than in invasive breast cancer. (Table 3).

### Table (3): Difference in RAGE expression parameters in relation to different stages of malignancy and lymph node metastatic deposits:

<table>
<thead>
<tr>
<th>STAGE</th>
<th>RAGE %</th>
<th>RAGE intensity</th>
<th>RAGE score</th>
<th>LN RAGE %</th>
<th>LN RAGE intensity</th>
<th>LN RAGE score</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCIS</td>
<td>Mean</td>
<td>4.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>1.41421</td>
<td>0.0000</td>
<td>0.0000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Low stage</td>
<td>Mean</td>
<td>36.6667</td>
<td>2.1772</td>
<td>5.3333</td>
<td>95.0000</td>
<td>5.0000</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>39.42207</td>
<td>8.843</td>
<td>5.7735</td>
<td>8.0901</td>
<td>2.3636</td>
</tr>
<tr>
<td>High stage</td>
<td>Mean</td>
<td>70.0000</td>
<td>2.0455</td>
<td>7.0000</td>
<td>85.0901</td>
<td>2.3636</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>49.45983</td>
<td>.71380</td>
<td>3.49085</td>
<td>8.44081</td>
<td>.48661</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td>P&lt;0.001</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

The tumor grade correlates significantly with both the RAGE intensity and RAGE score of both the primary tumor and the metastatic lymph node deposits. (P<0.05). The tumor stage on the other hand correlates positively with RAGE percent and score of primary tumor tissue (p<0.05) and correlates negatively with all parameters of RAGE expression in metastatic lymph node deposits (p<0.05)

RAGE percent of primary tumor tissue shows high significant correlation with RAGE percent of metastatic lymph node deposits (p< 0.01).
**Figures**

**Fig. (1A):** Section in case of benign breast lump diagnosed as fibrocystic disease if breast, showed breast ductules lined by hyperplastic ductal cells and surrounded by fibrous stroma (H&E stain, X200). **Fig. (1B):** Section in benign breast tissue showing dilated ducts mostly negative for RAGE expression (IHC for RAGE, X100).

**Fig. (2A):** Section in case of duct carcinoma in situ (DCIS), showing a cribriform pattern (H&E stain, X200). **Fig. (2B):** Section in case of DCIS showing mild RAGE expression. (IHC for RAGE, X100). **Fig. (3A):** Section in case of invasive duct carcinoma, showing solid sheets of malignant ductal cells invading the fibrous stroma (H&E stain, X200). **Fig. (3B):** Section in invasive duct carcinoma showing marked expression of RAGE within the cytoplasm of malignant cells. (IHC for RAGE, X200).
Fig.(4A): Section in case of invasive lobular carcinoma, showing infiltration of the fibrous stroma by small malignant cells arranged in thin rows “indian file pattern) (H&E stain, X200).

Fig. (4B): Section in case of invasive lobular carcinoma showing marked cytoplasmic expression of RAGE. (IHC for RAGE, X200).

Fig.(5A): Section in case of mucoid carcinoma of breast, showing groups of malignant ductal cells swimming in pools of mucin (H&E stain, X200).

Fig. (5B): Section in case of invasive tubular carcinoma of breast showing moderate expression of RAGE. (IHC for RAGE, X200).

Fig.(6A): Section in case of metastatic deposits of invasive duct carcinoma in an axillary lymph node (H&E stain, X200).

Fig. (6B): Section in axillary lymph node showing metastatic deposits of duct carcinoma with marked cytoplasmic expression of RAGE within the malignant cells. (IHC for RAGE, X200)

**Discussion:**

Breast cancer is a complex disease that results from the inheritance of a number of susceptible genes\(^1,2\). Although exhaustive investigations from single-locus to genome-wide association studies have been conducted, to unravel the ultimate genetic underpinnings of breast cancer still remains a challengeable task \([7]\).

Metastasis is a major cause of mortality in Breast Cancer (BC) patients. Among the different types of BC, triple negative BC (TNBC) (ER-, PR-, and HER2-) has been associated the most with poor prognosis and survival due to early metastasis to other organs and a lack of clinically established targeted therapies. Hence, elucidating novel mechanisms that regulate metastasis would lead to the development of targeted therapies and new treatments for TNBC and metastatic breast cancers \([2]\). It is now well accepted that solid tumors, including those in the breast, have an inflammatory microenvironment. Receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules which has been associated with chronic
inflammation, which in turn enhances the progression of various cancers [14].

**Our study** was conducted on breast and lymph node biopsies from 66 female patients ranging in age from 18 to 55 years. The mean age for benign cases was 31.45±9.22 years and for malignant cases was 48.65±13.81 (p <0.01). It is well documented that the risk of getting breast cancer increases with age. That is, 3 or 4 out of every 100 women who are 60 years old today will get breast cancer by the age of 70 [10]. Tissue samples include 6 excision biopsies from benign cases and 60 modified radical mastectomy biopsies from malignant cases. Benign lesions include fibrocystic disease of breast (4 cases) and fibroadenoma (2 cases), while malignant tumors include invasive duct carcinoma (50 cases), invasive lobular carcinoma (2 cases), medullary carcinoma (1 case), mucoid carcinoma (1 case) and intraduct carcinoma (6 cases). Invasive duct carcinoma cases include 3 cases of tubular carcinoma, and 47 cases of non-otherwise specified (NOS) carcinoma. Most of the studied cases were of low grade but high stage of malignancy (GI&II, T2&3).

It is now believed that most solid tumors, including those in the breast, have an inflammatory microenvironment [15, 6].

The selection of RAGE gene as a research candidate is biologically plausible. RAGE is a member of the immunoglobulin superfamily of cell surface receptors, and its interaction with advanced glycation end products and other molecules plays a role in the pathogenesis of cancer progression and metastasis [20]. There were high significant differences in means of all RAGE expression parameters between benign and malignant breast lesions, with higher levels achieved in malignant cases. This was in agreement with [9].

Low grade invasive breast cancer show higher percentage of RAGE expression compared to DCIS and high grade invasive breast cancer with significant difference between groups (p<0.01). RAGE intensity and RAGE score showed increasing values from DCIS up to high grade invasive breast carcinoma. However, the difference between groups was not statistically significant (p>0.05).

Our results were in concordance with the results of [9]. We found also that, the intensity and score of RAGE expression were higher in lymph node metastatic deposits of high grade invasive breast cancer compared to the same parameters in case of low grade invasive breast cancer with statistical significance (p<0.05 and p<0.01 respectively). On the other hand the percentage of RAGE expression in lymph node of metastatic deposits was non-significantly higher in low grade invasive breast cancer compared to cases of high grade invasive breast cancer (p>0.05). These results were in agreement with [14] showing that RAGE is preferentially expressed in invasive and lymph node metastasis tissues and with [16].

Our results were confirmed by the fact that the tumor grade correlates significantly with both the RAGE intensity and RAGE score of both the primary tumor and the metastatic lymph node deposits. (P<0.05). The tumor stage on the other hand correlates positively with RAGE percent and score of primary tumor tissue (p<0.05) and correlates negatively with all parameters of RAGE expression in metastatic lymph node deposits (p<0.05). RAGE percent of primary tumor tissue shows high significant correlation with RAGE percent of metastatic lymph node deposits (p<0.01). In evaluating the functional contributions of RAGE in breast cancer, found that RAGE-deficient mice displayed a reduced propensity for breast tumor growth. In an established model of lung metastasis, systemic blockade by injection of a RAGE neutralizing antibody inhibited metastasis development [14]. Our results give additional evidence that not only primary breast cancer but also metastatic breast cancer within axillary lymph nodes would be also a subject for anti-RAGE targeted therapy.

**References**


