ABSTRACT

Background: Breast cancer (BC) is the most common cause of malignancy in females all over the world. Continuous scientific research for the discovery of new markers helping is a cornerstone for early disease detection and proper management.

Aim of the Study: This study aimed to evaluate the role of Neutrophil gelatinase-associated lipocalin (NGAL) as prognostic markers for breast cancer in an Egyptian female population.

Patients and Methods: 120 BC patients and 30 healthy controls were the subjects of the study; serum NGAL levels were investigated and correlated with the clinicopathologic characteristics of the BC patients.

Results: Our study showed that NGAL was significantly different between healthy controls and BC patients, and it revealed a gradual increase with disease severity.
Conclusion: Our findings suggested that NGAL could be diagnostic marker for early case detection, and was shown to be associated with breast cancer prognosis, supporting its role as prognostic biomarker.

Keywords: Breast cancer; NGAL; diagnosis; prognosis.

1. INTRODUCTION

Recently, variable tumor marker levels in the serum have been approved as a diagnostic utility to detect the tumor activity. Tumor markers are considered as minimally invasive low cost indicators for follow up of the disease, its prognosis, and decision of treatment planning. Attention should be paid to the test benefits and limitations for achieving the best interpretation of results. The little diagnostic sensitivity of breast cancer at its early stage elicits questionable role of tumor markers [1].

No protein was established as a single biomarker for the BC screening at the present. Otherwise, the application of combined biomarkers is routinely used, such as CA15.3 and CEA [2], indicating the weakness of using each biomarker alone. However, this is costing and complicating the evaluation process. The need for identifying high diagnostic value biomarkers which can act singly in the screening and the detection of breast cancer was elicited.

Accordingly, for diagnostic, prognostic and predictive issues, the use of recently discovered BC biomarkers has been widely applied, such as Neutrophil gelatinase-associated lipocalin (NGAL).

NGAL (lipolacin 2) is a protein that was described in human neutrophil, evolved in bone marrow during the maturation of granulocyte [3]. Recently, it has been assessed in several physiological and pathological conditions as acute renal injury and variable types of human cancer as gastrointestinal tracts (GIT), liver, lung and thyroid cancers. Few studies have investigated its relation to BC [4].

Considering these facts, this study aimed to evaluate the role of NAGL as a biomarker in Egyptian females with BC and to evaluate the optimal cutoff values determining disease prognosis.

2. PATIENTS AND METHODS

2.1 Patients

2.1.1 Study design: This is a case control study

This study was performed in Zagazig university hospital, Zagazig, Egypt, from January 2019 to January 2020. This study was approved by Zagazig university ethics committee.

2.1.2 The study groups

- Group 1 (control): 30 healthy female subjects, with age and BMI matching to the patient group. These were female subjects that showed normal screening mammogram with no family history of breast cancer, no history of breast mass, pain, abnormal discharge or breast skin changes.

- Group 2 (patients): 120 breast cancer patients, with recently pathologically proved breast cancer. These patients were further classified into four subgroups according to the different disease stage (I, II, III and IV), and each group included 30 patients. The stages were defined according to TNM system as following:-

Stage I: T1N0M0.

Stage II: T0N1M0, T1N1M0, T2N0M0, T2N1M0 or T3N0M0.

Stage III: T0N2M0, T1N2M0, T2N2M0, T3N1M0, T3N2M0, T4N0M0, T4N1M0, T4N2M0 or any T N3M0.

Stage IV: any T any N M1.

Patients' clinical and pathological data [lesion size (T), node status (N), presence or absence of metastasis (M), tumor grading, ER, PR and HER2 results] were retrieved from patients medical records.

2.2 Methods

Study subjects serum samples were stored at -20 °C till the time of use. They were assayed for
serum NGAL levels according to the manufacturer’s instructions (BiovendorInc, Brno, Czech Republic):

- 100 µl of diluted Standards, Quality Controls, Dilution Buffer (=Blank) and samples were introduced in duplicates, into the appropriate wells.

- The plate was incubated at room temperature (25°C) for 1 hour, shaking was carried out at ca. 300 rpm on an orbital microplate shaker.

- The wells were washed 3-times with Wash Solution (0.35 ml per well). After final wash, the plate was inverted and tapped strongly against paper towel.

- 100 µl of Biotin Labelled Antibody solution was added into each well.

- The plate was incubated at room temperature (25°C) for 1 hour, shaking was carried out at ca. 300 rpm on an orbital microplate shaker.

- The wells were washed 3-times with Wash Solution (0.35 ml per well). After final wash, the plate was inverted and tapped strongly against paper towel.

- 100 µl of Streptavidin-HRP Conjugate was added into each well.

- The plate was incubated at room temperature (25°C) for 30 minutes, shaking was carried out at ca. 300 rpm on an orbital microplate shaker.

- The wells were washed 3-times with Wash Solution (0.35 ml per well). After final wash, the plate was inverted and tapped strongly against paper towel.

- 100 µl of Substrate Solution was added into each well.

- The plate was incubated for 10 minutes at room temperature.

- The color development was stopped by adding 100 µl of Stop Solution.

- The absorbance of each well was determined using a microplate reader set to 450 nm and corrected by subtracting the reading at 630 nm.

2.3 Statistical Analysis

MedCal_version 17.9.7 software was used for the analysis (MedCalc Software bib, Ostend, Belgium). Quantitative data were expressed as mean and standard deviation, while qualitative data were expressed as frequency and percentage. Nottingham prognostic index (NPI) values of the patients were calculated and interpreted [5]. Pearson tests were carried out for correlation of the serum marker with the clinical-pathological data of the patients. ROC curve analysis was done to estimate the cutoff point for differentiation between healthy subjects and breast cancer patients.

3. RESULTS

3.1 Age and Body Mass Index (BMI)

There was no significant age or BMI difference as control subjects were selected matching to BC patients in both parameters (Table 1).

3.2 Histopathological Type and Tumor Grade

The most prevalent histopathological type of BC (99 cases; 82.5%) was invasive ductal carcinoma (IDC). 9 cases (7.5 %) were invasive lobular carcinoma (ILC), 4 cases (3.34%) were mucinous carcinoma; 3 cases (2.5%) were medullary carcinoma, 3 cases (2.5%) were malignant phyllodes tumor, and 2 cases (1.66%) were poorly differentiated carcinoma. Regarding to the tumor grade, 12 patients were of grade I (10%), 79 patients were of grade II (65.8%) and 29 patients were of grade III (24.2%).

Table 1. Mean ± SD of women age and BMI among studied groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control group</th>
<th>Breast cancer group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>(Group I) n =30</td>
<td>(Group IIa) Stage I n=30</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.3 + 9.7</td>
<td>50.1+ 12.4</td>
</tr>
<tr>
<td>P</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>31.5 + 6.1</td>
<td>30.4+7.6</td>
</tr>
<tr>
<td>P</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>
3.3 NPI

The breast cancer patients were reclassified according to NPI values, most of the patients were of moderate prognosis group (Table 2).

3.4 Serum NGAL Levels

Step rise increase in the serum NGAL levels as the patient's stage progress is evident in Table 3, with high significant difference (p < 0.01) could be noted between control subjects and breast cancer group as well as between the stage III and stage IV patients.

Pearson correlation testing of the serum NGAL with the clinicopathological characteristics of the patients is shown in Table 4 and Figs. 1, 2, 3 & 4 which revealed that NGAL levels were showing a non-significant correlation with the age, while a significant correlation was noted with the patient NPI values and a highly significant correlation was noted with tumor size, node status and histopathological grade.

Table 2. The breast cancer patients prognosis according to the NPI values

<table>
<thead>
<tr>
<th>Patients prognosis according to NPI</th>
<th>N</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Excellent prognosis.</td>
<td>2</td>
<td>1.67%</td>
</tr>
<tr>
<td>- Good prognosis.</td>
<td>19</td>
<td>15.83%</td>
</tr>
<tr>
<td>- Moderate prognosis.</td>
<td>75</td>
<td>62.5%</td>
</tr>
<tr>
<td>- Poor prognosis.</td>
<td>24</td>
<td>20%</td>
</tr>
<tr>
<td>* Total</td>
<td>120</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 3. The mean values of serum NGAL in the groups of the study

<table>
<thead>
<tr>
<th>Mean±SD</th>
<th>C</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGAL (ng/mL)</td>
<td>117.4±15.7</td>
<td>332±60.6</td>
<td>325±43.6</td>
<td>335.3±62.6</td>
<td>410.6±112.7</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.01</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Table 4. Correlations between the serum NGAL levels and different clinic-pathological parameters in the breast cancer patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Tumor size</td>
<td>0.42</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Node status</td>
<td>0.45</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tumor grade</td>
<td>0.35</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>NPI values</td>
<td>0.4</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Fig. 1. Correlation between serum NGAL level and tumor size
Fig. 2. Correlation between serum NGAL level and node status

Fig. 3. Correlation between serum NGAL level and tumor grade

Fig. 4. Correlation between serum NGAL level and NPI
In this study, the serum NGAL cutoff value to
differentiate healthy controls from breast cancer
patients was 277.9 ng/mL, the calculated
sensitivity and specificity were 83% and 100%
respectively.

4. DISCUSSION

Breast cancer is highly heterogeneous in terms
of its etiology and pathological characteristics [6],
some cases are showing slow growth with an
excellent prognosis, whereas other cases are
taking a highly aggressive clinical course. Much
effort is made on the scientific, economic, and
organizational levels for better understanding of
the eliciting factors, the molecular motivations for
progression and the best effective, least
hazardous intervention lines [7]. Serum levels of
NGAL in breast cancer patients are recently
considered as predictive and prognostic
indicators for the disease [8,9]. This study is a
case control study and included 150 subjects, of
which 30 are healthy controls and 120 are BC
patients who were admitted to the oncology
department of Zagazig University Hospital,
Egypt. This study aimed to evaluate the
diagnostic and prognostic role of NAGL
biomarker in Egyptian female patients with BC.

The glycoprotein NGAL/ Lipocalin 2 has been
originally proposed for early pick up of acute
renal injury states [10]. However, some
limitations of its use have been reported [11].

NGAL role in oncological process has been
growingly evidenced. Interestingly, NGAL shows
both up and down regulation depending on the
type of the malignancy [12]. Scientists have
begun focusing on NGAL biomarker assessment
as a novel simple, non-complicated, easily
accessible, non-invasive method for cancer
diagnosis and prognosis, owing to its availability
of being detected in both urine and blood [12].

NGAL is proved to be related to the regulation of
epithelial mesenchymal transition (EMT), which
is known to be incorporated in BC progression
[13,14]. Some studies have proposed that NGAL
leads to apoptosis and suppression of the
proliferation process [15,16]. Meanwhile, others
have concluded that the NGAL can stimulate
tumor proliferation and invasion [17,18]. In this
study, the NGAL levels in serum were
investigated. The levels were differing
significantly between control subjects and BC
patients and between stage III and stage IV BC
patients. The results of this study revealed that

One previous systematic review revealed the
overall NGAL diagnostic and prognostic value in
breast cancer. In a different study, as in the
present one, a relation was proposed between
the higher NGAL levels and BC poor prognosis
[19]. Many other studies also showed similar
results [20,21,22].

A previous study included females with
pathologically proved non-palpable breast
carcinomas and 30 healthy females acting as
controls. Notably, the NGAL showed significantly
higher levels in BC patients when compared with
control subjects [20]. Another study reported
positive correlation of NGAL with tumor grade
and N stage [23]. Li et al study has concluded
the association between NGAL levels and BC
patients' poor prognosis [24].

The limitations of this study were that no patients
follow up was performed and the survival rates
were not evaluated. However, the study has
several strength points, among them are that, to
the best of our knowledge, this marker was not
evaluated before in Egyptian population and no
cutoff value was proposed in them for reliable
diagnosis and prognosis of breast cancer
disease.

5. CONCLUSION

Our findings suggested that NGAL could be
diagnostic marker for early cases detection, and
it revealed association with the BC prognosis,
NGAL serum level has shown to have a step rise
increase as the disease stage get worse,
ensuring its value as prognostic biomarkers.

COMPETING INTERESTS

Authors have declared that no competing
interests exist.

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