Original Research Article

Study of the Potential Role of Serum Neutrophil Gelatinase-Associated Lipocalin (NGAL) Levels in the Diagnosis and Prognosis of Breast Cancer in Egyptian Females "a Case-Control Study".

ABSTRACT:

Background: breast cancer (BC) is the most common cause of malignancy in females all over the. Continuous scientific research for the discovery of new markers helping is a cornerstone for early disease detection and proper management. The 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. The results: our study showed that NGAL is significantly differs 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.

KEY WORDS:

Breast cancer, NGAL, diagnosis, prognosis.

INTRODUCTION:

Recently, variable tumor marker levels in the serum have been approved as a diagnostic utility the tumor activity detection. 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.

PATIENTS AND METHODS

Patients

- Study design: this is a case control study.

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

The study groups:

- Group 1 (control): 30 healthy female subjects, age and BMI matches 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 that were, with recently pathologically proved breast cancer, these were further classified into four subgroups according to the different disease stages (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 retrived from patients medical records.

Methods

Study subjects serum samples, that were -20 were assayed for serum NGAL levels according to the manufacturer's instructions (Biovendor Inc,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 werewashed 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 (acceptable range: 550 - 650 nm). readings were substracted at 630 nm (550 - 650 nm) from the readings at 450 nm.

Statistical analysis

MedCal_version 17.9.7 software was used for the analysis of the (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.

RESULTS:

Age and body mass index (BMI)

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

Groups / **Parameter**	**Control Group**	**Breast Cancer Group**			
	(Group I) n =30	(Group IIa) Stage I n=30	(Group IIb) Stage II n=30	(Group IIc) Stage III n=30	(Group IId) Stage IV n=30
Age (years)	48.3 + 9.7	50.1+ 12.4	49.3+9.9	48.9 +10.2	50.3+11.1
P		>0.05	>0.05	>0.05	>0.05
BMI (kg/m2)	31.5 + 6.1	30.4+7.6	30.8+6.6	31.3 + 6.4	29.2+ 11.4
P		>0.05	>0.05	>0.05	>0.05

Histopathological type and tumor grade

The most prevalent histolopathological 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%).

NPI (table 2)

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

Patients prognosis according to NPI	**n**	**Percentage**
- Excellent prognosis.	2	1.67%
- Good prognosis.	19	15.83 %
- Moderate prognosis.	75	62.5%
- Poor prognosis.	24	20%
* Total	120	100%

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 and between the stage III and stage IV patients.

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

Mean±SD	**C**	**Stage I**	**Stage II**	**Stage III**	**Stage IV**
NGAL (ng/mL)	117.4±15.7	332±60.6	325±43.6	335.3±62.6	410.6±112.7
p		<0.01	>0.05	>0.05	<0.01

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

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

Variables	NGAL	
	R	p
Age	0.1	>0.05
Tumor size	0.42	<0.01
Node status	0.45	<0.01
Tumor grade	0.35	<0.01
NPI values	0.4	<0.05

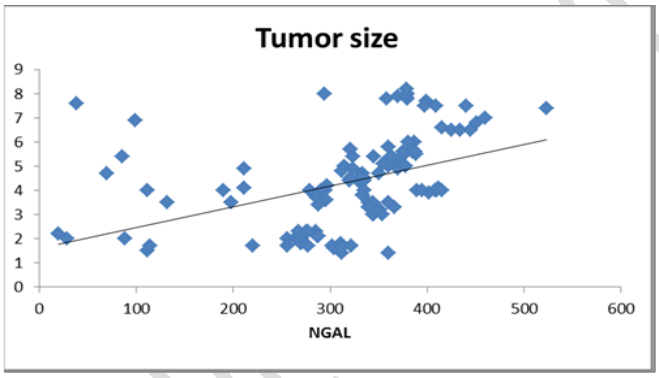

Figure 1: Correlation between Serum NGAL level and tumor size.

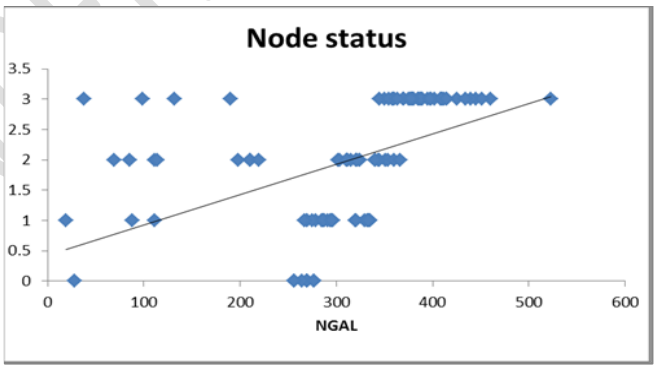

Figure 2: Correlation between Serum NGAL level and node status.

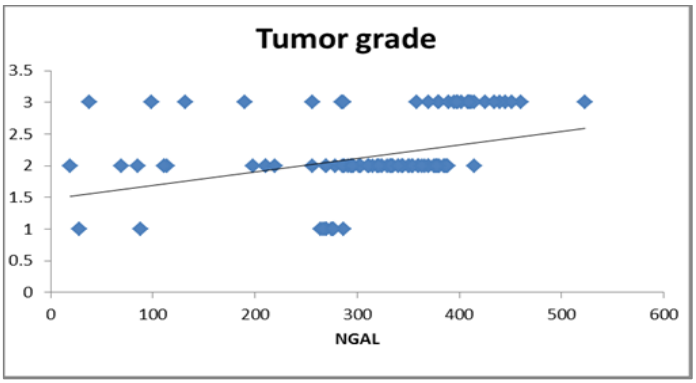

Figure 3: Correlation between Serum NGAL level and tumor grade.

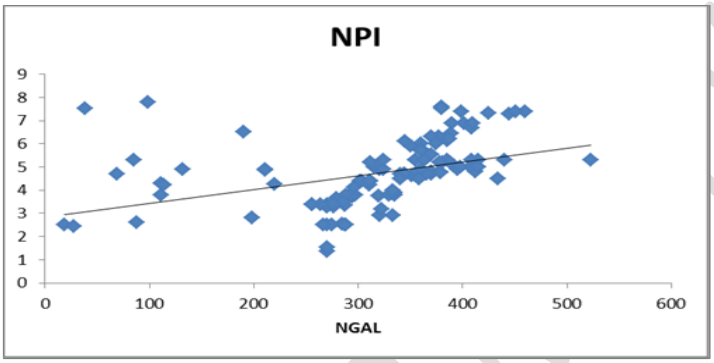

Figure 4: Correlation between Serum NGAL level and NPI.

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.

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 biomarkers 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 of its use limitations 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 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 serum NGAL levels were showing no significant correlation with patients' age, while showing significant/high significant correlation with tumor size, node status, tumor grade and accordingly the NPI status. 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.

One previous systematic review revealed the overall NGAL diagnostic and prognostic value in breast cancer. As with our study, in that study, 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.

CONCLUSION

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

REFERENCES

1. Banegas P, Y. Bird J, Moraros S, King S, Prapsiri B and Thompson p: Breast cancer knowledge, attitudes, and early detection practices in United States-Mexico border Latinas, Journal Women's Health, 21 (1) 2012;(3):101-107.

2. Nicolini A, Carpi A, Ferrari P and Rossi G: Immunotherapy prolongs the serum CEA-TPA-CA15.3 lead time at the metastatic progression in endocrine-dependent breast cancer patients: a retrospective longitudinal study. Cancer Lett. 2008; 263: 122-129.

3. Flower DR: The lipocalin protein family: structure and function. Biochem J. 1996; 318 (Pt 1): 1-14.

4. Wang YU and Zeng T: Neutrophil gelatinase associated lipocalin protein as a biomarker in the diagnosis of breast cancer: A meta analysis. Biomedical Reports. 2013; 1: 479-483.

5. Garner J: "In the case of breast cancer, how can these be combined to give prognostic information?". Questions for the MRCS vivas. 2004; London: Arnold. p. 231.

6. Abdel-Aziz T, Azab N, Emara N, Odah M and I.M. El-deen: Study of BRCA2 Gene Mutations in Egyptian Females with Breast Cancer. International Journal of Innovative Research in Science. 2015; 4(2): 283-288.

7. Fryczkowski M, Bułdak J, Hejmo T, Kukla M and Żwirska-Korczala K: Circulating levels of NGAL, leptin, VEGF, and HGF and their clinical relevance with PSA marker in prostate cancer. Disease markers, 2018;3852401:9-21.

8. Kim Y, Seok JY, Hyun KY, Lee GH, and Choi SC: Correlation of Glasgow pronostic score or procalcitonin to clinical variables in patients with pretreatment lung cancer. Biomedical Science Letter. 2016; 22: 9-17.

9. Kurozumi S, Alsaeed S and Orah N: Clinicopathological significance of lipocalin 2 nuclear expression in invasive breast cancer. Breast Cancer Res Treat. 2020; 179, 557–564.

10. Soni SS, Ronco C, Katz N and Cruz DN: Early diagnosis of acute kidney injury: the promise of novel biomarkers. Blood Purif. 2009; 28(3): 165–174.

11. Devarajan P: Review: neutrophil gelatinase-associated lipocalin: a troponin-like biomarker for human acute kidney injury. Nephrology (Carlton). 2010; 15(4): 419–428.

12. Roli L, Pecoraro V and Trenti T: Can NGAL be Employed as Prognostic and Diagnostic Biomarker in Human Cancers? A Systematic Review of Current Evidence. The International Journal of Biological Markers. 2017; 32(1), 53–61.

13. Wu Y, Sarkissyan M and Vadgama JV:Epithelial-mesenchymal transition and breast cancer. J Clin Med. 2016; 5:E13.

14. Masuda H, Baggerly KA and Wang Y: Differential response to neoadjuvant chemotherapy among 7 triple-negative breast cancer molecular subtypes. Clin Cancer Res. 2013; 19: 5533–5540.

15. Wang YP, Yu GR and Lee MJ: Lipocalin-2 negatively modulates the epithelial-to-mesenchymal transition in hepatocellular carcinoma through the epidermal growth factor (TGF-beta1)/ Lcn2/Twist1 pathway. Hepatology. 2013; 58:1349–1361.

16. Leung L, Radulovich N and Zhu CQ: Lipocalin2 promotes invasion, tumorigenicity and gemcitabine resistance in pancreatic ductal adenocarcinoma. PLoS ONE. 2012; 7(10): e46677.

17. Feng M, Feng J and Chen W: Lipocalin2 suppresses metastasis of colorectal cancer by attenuating NF-κB-dependent activation of snail and epithelial mesenchymal transition. Mol Cancer. 2016; 15: 77.

18. Leng X, Ding T and Lin H: Inhibition of lipocalin 2 impairs breast tumorigenesis and metastasis. Cancer Res. 2009; 69: 8579–8584.

19. Wang YU and Zeng T: Neutrophil gelatinase associated lipocalin protein as a biomarker in the diagnosis of breast cancer: A meta analysis. Biomedical Reports. 2013; 1: 479-483.

20. Provatopoulou X, Gounaris A, Kalogera E, Zagouri F, Flessas I, Goussetis E, Nonni A, Papassotiriou I and Zografos G: Circulating levels of matrix metalloproteinase-9 (MMP-9), neutrophil gelatinase-associated lipocalin (NGAL) and their complex MMP-9/NGAL in breast cancer disease. BMC Cancer. 2009; 9(390).

21. Yang J, Bielenberg DR, Rodig SJ, Doiron R, Clifton MC, Kung AL, Strong RK, Zurakowski D and Moases MA: Lipocalin 2 promotes breast cancer progression. PNAS. 2009; 106: 3913-8.

22. Candido S, Maestro R, Polesel J, Catania A, Maira F, Signorelli S, McCubrey J and Libra M: Roles of neutrophil gelatinase-associated lipocalin (NGAL) in human cancer. Oncotarget. 2014; 5(6).

23. Bauer M, Eickhoff JC, Gould MN, Mundhenke C, Maass N and Friedl A: Neutrophil gelatinase-associated lipocalin (NGAL) is a predictor of poor prognosis in human primary breast cancer. Breast Cancer Res Treat. 2008;108(3): 389–397.

24. Li SH, Hawthorne VS, Neal CL, Sanghera S, Xu J, Yang J, Guo H, Steeg PS and Yu D: Upregulation of neutrophil gelatinase-associated lipocalin by ErbB2 through nuclear factor-kappaB activation. Cancer Res. 2009; 69: 9163-8.