Significance of Serum HGF, Bcl-2 and Nitric Oxide in Primary Breast Cancer

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Abstract: The aim of this study was to determine serum concentrations of HGF, Bcl-2 and nitric oxide (NO) in 44 patients with primary breast cancer and 15 healthy individuals as a control group using an ELISA assay for HGF and Bcl-2 while nitric oxide was determined by using colorimetric technique. The measured parameters were correlated with clinicopathological parameters that may affect the outcome of disease. In addition, ROC curve analysis was done to each parameter. The results were as follows, the mean level of HGF was 1198.79 ± 76.32 pg/ml compared with 884.67 ± 66.88 pg/ml for control (p = 0.026). The HGF levels were significantly elevated in the patients with increasing the tumor stage (p = 0.036). In addition, HGF levels were higher in negative estrogen receptor (p = 0.039). The mean level of Bcl-2 in patients was 12.83± 1.97 ng/ml compared with 5.09 ± 0.40 ng/ml for control (p = 0.027). Levels of Bcl-2 were elevated but not statistically significant in patients with GI tumors, negative nodes, ER negative tumors and postmenopausal patients (p = 0.4, 0.8, 0.7 and 0.5, respectively).The patients mean level of the nitric oxide (NO) was 63.07 ± 4.14 µmol/L compared with 43.99 ± 4.21 µmol/L for control (p = 0.014). The levels of NO were elevated but also not statistically significant in patients with tumor size I, GI tumors, ER negative tumors, positive nodes, stage II tumors and postmenopausal patients (p = 0.3, 0.6, 0.3, 0.7, 0.3 and 0.2 respectively). From the ROC curve analysis, it was observed that the area under curve for HGF, Bcl-2 and NO was 0.695, 0.842 and 0.711, respectively. This result indicates the good validity of the above markers especially Bcl-2 parameter to discriminate the positive from the negative samples. In conclusion, this study demonstrates that the serum determination of HGF, Bcl-2 or NO may help in diagnosis of the breast cancer and may aid in disease prognosis. However, larger studies with more patients are required.
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Key words: HGF; Bcl-2; nitric oxide; breast cancer; diagnosis; prognosis.

Introduction

Breast cancer is the second most common cancer in the world, and is the most common cancer in women [1]. In excess of 1.2 million cases are detected every year, affecting 10–12% of women and responsible for approximately 500,000 deaths per year [2]. The ability to detect human malignancy via a simple blood test has long been a major objective in medical screening. The advantages of such an easy to use, relatively non-invasive and operator-independent test are self-evident. In this respect, cancer biomarkers can be DNA, mRNA, proteins, metabolites, or processes such as apoptosis, angiogenesis or proliferation [3].

HGF is a cytokine which induces morphogenesis, proliferation, motility and angiogenesis [4]. In normal mammary development, HGF in collaboration with other growth factors such as neuregulin stimulates tubulogenesis in a tightly controlled paracrine manner [5]. HGF is primarily expressed by mesenchymal/stromal cells, whereas its receptor, Met, is expressed selectively by epithelial cells, thereby creating a paracrine regulatory system [6]. In normal breast tissue, the HGF-and-Met paracrine system has a low basal level of expression [7]. Over-expression of Met [8] and HGF [9] in breast tumors and of HGF in the sera [10] of breast cancer patients has been found to be an independent predictor of recurrence and decreased patient survival. HGF has antiapoptotic effects [11]. Recent studies also suggest that HGF suppresses cell apoptosis by up regulating the expression of Bcl-xl, an antiapoptotic protein [12]. The suppression of apoptosis contributes to carcinogenesis, as well as to a resistance to chemotherapy and radiotherapy [13]. Apoptosis appears to be controlled by several genes. A group of genes with sequences homologous to bcl-2 modulate cell death and can be divided into two functionally antagonistic groups: suppressors, such as Bcl-2, and cell promoters, such as Bax. Homo or heterodimerization are important for the apoptotic regulatory function of the bcl-2-related proteins. The ratio between Bax/Bcl-2 heterodimers appears to be essential in deciding the life or death of a cell. When Bax predominates, apoptosis is accelerated and the antiapoptotic activity of Bcl-2 is antagonized [14, 15].

B-cell lymphoma leukemia-2 (Bcl-2) protein is a member of the bcl-2 family that regulates apoptosis [16] and is expressed in normal glandular epithelium. It's tumourigenic potential has been demonstrated in animal models [17] and is supported by the finding of

over expression of Bcl-2 in a variety of tumors and in lymphomas in which Bcl-2 acts as an oncogene [18]. It is over expressed in 25–50% of breast cancers [19]. High expression of Bcl-2 is considered as a good prognostic factor in cancer since it has been associated with improved survival in patients with breast cancer [20, 21]. High expression of Bcl-2 has been observed in ER-positive breast cancers as well as in progesterone receptor (PR) - positive breast cancers [22, 23].

Nitric oxide (NO) is a free radical acting as a gaseous messenger that affects various biological functions, either at low concentrations as a signal transducer in many physiological processes (e.g., blood flow regulation, smooth muscle relaxation, iron homeostasis, platelet reactivity, neurotransmission) or at high concentrations as a cytotoxic defensive mechanism against pathogens and, perhaps, tumors [24]. Moreover, accumulating evidence suggests that chronically elevated levels of NO are involved in the pathogenesis of some human pathological conditions, such as cancer [25]. NO production is a part of the angiogenic switch in tumor development [26, 27].

NO may promote tumor growth by modulating the production of prostaglandins. NO can activate cyclooxygenase-2 (COX-2) [28, 29] that, by generating prostaglandins, promotes angiogenesis and inhibits apoptosis (e.g., by enhancing Bcl-2 protein synthesis) [30]. The aim of the present work is to determine serum levels of HGF, Bcl-2 and nitric oxide in patients with primary breast cancer and to correlate these parameters with the clinicopathological data of the patients which may affect the outcome of disease. This may help in distinguishing subsets of breast cancer patients and optimizing therapeutic approaches.

Patients and Methods

Forty-four patients with primary invasive breast cancer were included in this study. All the patients met the following criteria: (a) having been diagnosed as having primary invasive breast cancer, (b) having no clinical manifestation of infection, (c) having no other known malignancy. All the 44 patients were women ages 23 to 56 years (median, 36 years). Also, a group of 15 healthy females was used as control. The diagnosis was carried out by biopsy and imaging studies. The data of primary tumor stage, age, estrogen receptor status, progesterone receptor status, tumor size, lymph node status and histological grade were collected. Venous blood samples were collected before the surgery. Sera were obtained by centrifugation and stored at -70 ºC until assayed.

Determination of Serum HGF and Bcl-2 by ELISA

Circulating HGF and Bcl-2 were evaluated by solid-phase Enzyme-linked immunosorbent assay (RayBiotech, Inc and Bender MedSystems GmbH, Europe, respectively) using 96-well microplates in accordance with the manufactures instruction. The color conducted is stopped with stop solution, and the optical density was measured at 450 nm and reference filter 620 nm. A standard curve was constructed by plotting the mean absorbance obtained from each standard against its concentration. The best fit curve through the points of the graph was drawn. From these standard curves, the concentrations of HGF and Bcl-2 for the patients and control under the study were obtained. The concentrations read from the standard curve of Bcl-2 was multiplied by the dilution factor (x 5) due to1:5 dilution of the samples. Detection limit for HGF was less than 8 pg/ml while that of Bcl-2 was less than 0.5 ng/ml.

Nitric oxide Measurement in serum by Colorimetric Method

Quantitative estimation of nitric oxide in serum was carried out colorimetrically according to the method of Montgomery and Dymock [31], using Biodiagnostic nitric oxide kit (Egypt). The principle of the test is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by the colorimetric detection of nitrite as a deep purple azo compound.

Statistical Analysis

Data were expressed as mean ± SEM and were analyzed using Medcal software, version 11 by selecting The Student's t test was used to assess the significance of difference in the levels of HGF, Bcl-2 and NO between the patients group and the control group. One-way ANOVA was performed to differentiate the parameter within the same group of clinical data. The cut-off value was determined for each of the measured serum parameters in that study according to the best discrimination between patients and control regarding optimal values of sensitivity and specificity using ROC curves analysis. AUC of the tests using ROC curve was calculated. $P < 0.05$ was accepted as significant.

Results

The characteristics of the patients are shown in Table 1. The median age was 36 (23–56) years. All patients were with invasive ducal carcinoma, of which 7 (15.9 %) with grade I, 29 (65.9 %) with grade II and 8 (18.2 %) with grade III. Thirty-two patients were Premenapausal (72.7 %) and 12 were postmenopausal (27.3 %). The mean and standard error of mean (SEM) for serum HGF, Bcl-2 and NO levels in patients with breast cancer and control were illustrated in Table 2. Serum HGF concentrations of the breast cancer patients showed significant increase when compared with those of the control (1198.79 pg/ml versus 884.67

pg/ml, respectively, p = 0.026). There was also, significant increase in Bcl-2 serum level in breast cancer patients when compared with those of the healthy control (12.83 and 5.09 ng/ml, respectively, p = 0.027). In addition, Serum NO level revealed significant increase in patients with breast cancer when compared to those of the control (63.07 and 43.99 µmol/L, respectively, p = 0.014).

Table 1. Patient's characteristics.

Parameter	N	%
Age Median	36 (23–56)	
Tumor size		
T1 <2	26	59.1
T2 2–5	11	25
T3 >5	7	15.9
Auxiliary lymph node		
Positive	28	63.64
Negative	16	36.36
Clinical stage		
Stage I	28	63.64
Stage II	16	36.36
Pathological grade		
Grade I	7	15.9
Grade II	29	65.9
Grade III	8	18.2
Estrogen receptor		
Positive	18	40.9
Negative	26	59.1
Progesterone receptor		
Positive	19	43.2
Negative	25	56.8
Menopausal status		
Premenopausal	32	72.73
Postmenopausal	12	27.27

Table 2. The mean serum levels of HGF, Bcl-2 and Nitrate + Nitrite of the patients compared with those of control.

	HGF(pg/ml)	Bcl-2(ng/ml)	Nitrate + Nitrite (µmol/L)
Patients	1198.79 ± 76.32	12.83± 1.97	63.07 ± 4.14
Control	884.67 ± 66.88	5.09 ± 0.40	43.99 ± 4.21
P versus control	0.026*	0.027*	0.014*

Data were expressed as mean ± standard error. (*) significant

Correlations of Serum HGF, Bcl-2 and NO with Clinicopathological Parameters of Patients

Table 3 showed that there was significant elevation of HGF level in sera of the patients with negative estrogen receptor (p = 0.039) compared with that of patients with positive receptors. Also, there was significant elevation of HGF level in sera of the patients with clinical stage II (p = 0.036) compared with that of patients with clinical stage I. On the other hand, Table 3 showed that there was decreasing in the serum Bcl-2 mean level of the patients with increasing the grade, but the difference was not statistically significant (p = 0.4). In addition, there was insignificant increase in the Bcl-2 serum levels in the postmenopausal patients compared with those of premenopausal patients (p = 0.5). Also, table 3. illustrated that there is not significant variation of the nitric oxide levels with the clinicopathological data of the patients.

ROC Curve Analysis

The receiving operating characteristic (ROC) curve was designed for HGF, Bcl-2 and NO (Figures 1, 2 and 3). The cut-off value for HGF, Bcl-2 and NO was >1110, >5.5 and >60, respectively. It was found that the area under curve (AUC) for HGF, Bcl-2 and NO was 0.695, 0.842 and 0.711, respectively. This result indicates the good validity of the above markers especially Bcl-2 parameter to discriminate the positive from the negative samples.

Table 3. Correlations of HGF, Bcl-2 and NO with clincopathological data of patients.

Parameter	HGF(pg/ml)	Bcl-2(ng/ml)	NO (μmol/L)
Tumor size			
T1<2 cm	1147.95 ± 82.48	11.93±2.61	67.34 ± 4.65
T2 2–5 cm	1145.46 ± 171.21	15.36±4.82	61.76 ± 11.59
T3>5 cm	1471.43± 256.12	12.207±2.43	49.28 ± 6.35
Pathological grade			
Grade I	1230.57 ± 146.35	18.50±7.67	72.51 ± 9.48
Grade II	1103.75 ± 92.33	12.33±2.32	62.36 ± 5.40
Grade III	1515.5 ± 194.89	9.66±1.80	57.37 ± 8.50
Estrogen receptor			
Positive	1011.33 ± 110.95	11.94±2.47	57.68±7.18
Negative	1328.57 ± 97.53*	13.44±2.89	66.80±4.92
Progesterone receptor			
Positive	1201.79±126.85	11.989±3.05	54.85± 4.85
Negative	1196.51±95.89	13.470±2.62	69.32± 6.06
Menopausal status			
Premenopausal	1216.65±94.71	12.08±2.09	66.27±5.14
Postmenopausal	1151.17±125.69	14.83±4.71	54.54±6.16
Auxiliary lymph node			
Positive	1111.67±91.36	12.45±2.39	64.33±4.66
Negative	1351.251±131.11	13.50±3.53	60.87±8.13
p-value			
Clinical stage			
Stage I	989.38±97.85	12.40±2.46	59.62±5.12
Stage II	1318.46±100.39*	13.59±3.39	69.11±6.96
p-value			

Data were expressed as mean ± standard error
(*) significant

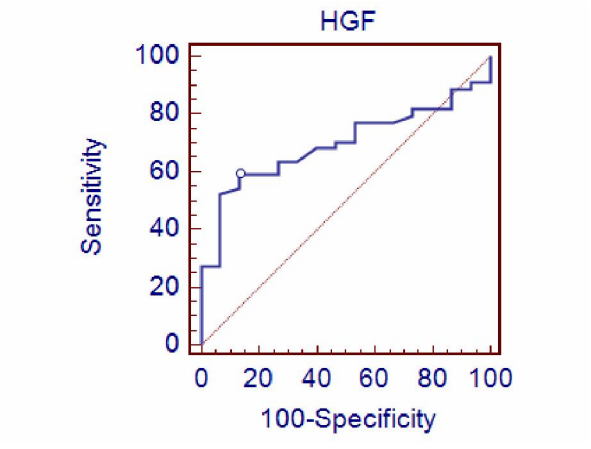

Figure 1. ROC curve of HGF, area under curve equal 0.695, $p = 0.004$.

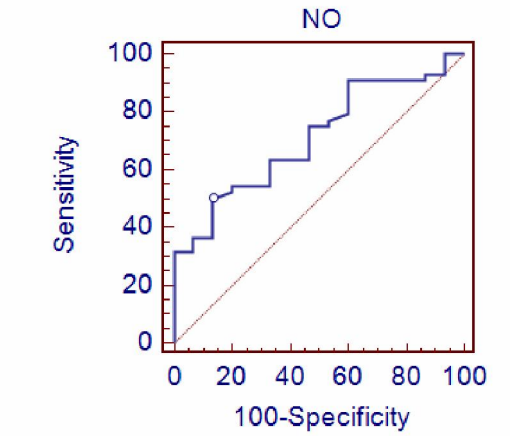

Figure 2. ROC curve of Bcl-2, area under curve equal 0.842, $p = 0.0001$.

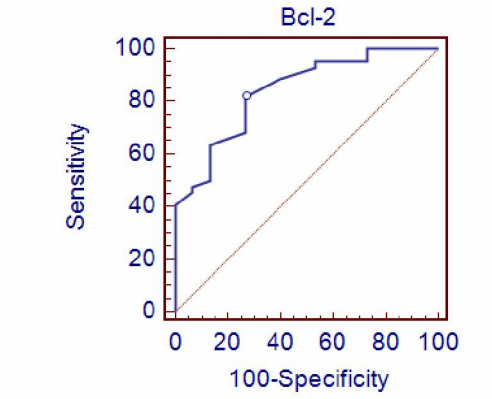

Figure 3. ROC curve of NO, area under curve equal 0.711, $p = 0.0036$.

Discussion

Biomarkers accepted for clinical use in breast cancer, such as CA 15-3, CEA and CA 27-29, have low sensitivity and specificity, and are thus more useful for patients at an advanced stage of breast cancer rather than for early cancer diagnosis [32]. So, there is a needing for new parameters to help in diagnosis and prognosis of primary breast cancer. So, in the present study, serum HGF, Bcl-2 and NO were evaluated and correlated with the clinicopathological parameters. Hepatocyte growth factor was originally identified both as a mitogen for parenchymal liver cells and as a fibroblast-secreted protein responsible for inducing the scattering of polarized epithelial cells (hence the alternative name, scatter factor). HGF and its receptor Met, a tyrosine kinase mediated product of the c-met proto-oncogene, are involved in a number of physiological activities, including cell proliferation, motility and migration, and invasion. In tumors, HGF disrupts adherens junctions and promotes cell dispersal, so stimulating invasive capacity [33].

It was reported that HGF receptor is widely distributed in various epithelial cells including tumor cells but obviously not in mesenchymal cells [34]. On the other hand, HGF production was found in the stromal component but not in the epithelial component of the breast [35]. Because it has been reported that HGF is a modulator of epithelial cell proliferation and motility for a broad spectrum of cell types [36], it is tempting to speculate that HGF originating from breast stromal cells may play a crucial role in facilitating breast cancer cell invasion and metastasis.

HGF is a mitogen for vascular endothelial cells (VECs) [37]. Maejima et al. [37] showed that the proliferative effect was partly dependent on nitric oxide synthesis, which was itself regulated by Src family kinases. Bell et al. [38] found that HGF secreted from adipose cells is involved in local angiogenesis, and specifically in the migration of VECs. Other investigators have demonstrated the induction of VEC protease production by HGF [39], and a consequent stimulation of VEC migration [40] and endothelial tube formation [38].

In the present study, it was observed that HGF serum levels were significantly elevated in the patients compared to those of control (p =0.026). Furthermore, there were significantly higher serum levels of HGF in patients with higher tumor stage (p = 0.036). Thus, the preoperative level of serum HGF may reflect the severity of invasive breast cancer and may be useful to pick up higher risk patients for more aggressive treatment. This result is consistent with Sheen-Chen et al. [41] who indicated that the mean value of serum soluble HGF in patients with invasive breast cancer was higher than that of the control group and the difference was significant, and concluded that patients with more advanced TNM staging were shown to have higher serum soluble HGF and the preoperative serum soluble HGF levels might reflect the severity of invasive breast cancer.

There are studies suggest that HGF suppresses cell apoptosis by up regulating the expression of Bcl-xl, an antiapoptotic protein [12]. Bcl-2 is a cytoplasmic protein involved in apoptosis and oncogenesis; it prolongs the survival of the non-cycling cells and inhibits cycling cells [42]. During the developmental period, bcl-2 is expressed in all tissues, while in adults it is expressed only in proliferating or reserve cells [43]. As far as breast cancer is concerned, bcl-2 protein is generally expressed in 60–80% of invasive breast carcinoma [44, 45]. In breast cancer specimens, bcl-2 expression is associated with well-differentiated tumors, like lower SBR grade, ER positivity and a low proliferation status [46, 47]. Several studies suggested that the low apoptotic response caused by over expression of bcl-2 allows the accumulation of genetic alterations that might be important in metastatic breast cancer potential [48, 49]. Bcl-2 expression has been reported to be associated with better outcomes in metastatic disease as well as in patients with early breast cancer treated with either hormone or chemotherapy [50, 51].

In the present study, Bcl-2 levels were significantly elevated (p = 0.027) in patients with breast cancer compared with those of the healthy control. These results agree with the finding of Kallel-Bayoudh et al. [52] who reported that Bcl-2 expression seems to be a very useful factor that should be in combination with HER2 and ER in breast cancer prognosis.

A strong inverse correlation between Bcl-2 and proliferative activity has been reported to exist in breast cancer, as well as in other tumor types, and the data presented in this study are in line with these findings, as the mean level of bcl-2 in Grade I, II and III was 18.50±7.67, 12.34±2.32 and 9.66±1.81, respectively.

Typically, tumors with low Bcl-2 expression are correlated with high grade histological type, indicating the existence of rapid cell turnover. In fact, similar relationships between apoptosis, proliferation and high tumor grade have been reported for other tumor types [53].

In breast carcinoma patients, a positive correlation between the expression of inducible NOS and metastatic disease has been reported by Martin et al. [54]. Elevated levels of NO production increase tumor vascularity and facilitate tumor metastasis in breast carcinoma patients [55]. NO may promote tumor growth by modulating the production of prostaglandins. NO can activate cyclooxygenase-2 (COX-2) [28, 29] that, by generating prostaglandins, promotes angiogenesis and inhibits apoptosis (e.g., by enhancing Bcl-2 protein synthesis) [30]. Conversely, another study suggested that NO inhibits the proliferation of human breast carcinoma cells, which explains the relationship between NO production and weak tumor aggressiveness [56]. Guntel et al. [57] found elevated level of nitrate+nitrite at operable serum in samples of patients with breast cancer.

In the current study, serum nitrate and nitrite levels showed significant increase in patients (p=0.014) compared with control subjects. These elevated nitrate and nitrite levels in the patients may be a result of increased NOS II activity, which is stimulated by a host defense system against tumor growth. Martin et al. [58] showed that endothelial NO synthetase activity was expressed by human breast tumors. NO synthetase is responsible for the production of NO. Increased NO synthetase activity is necessary for VEGF to stimulate angiogenesis and increase vascular permeability [59]. In addition, no correlation was found between nitrate + nitrite levels, and the prognostic factors of the breast tumor include tumor size, stage and menopausal status.

By ROC curve analysis, the area under (AUC) for HGF, Bcl-2 and NO was 0.695, 0.842 and 0.711, respectively. This indicates the availability of using these parameters in combination with the routine tumor markers such as CA 15.3 to help in diagnosis of breast cancer.

In conclusion, measurement of HGF, bcl-2 and nitric oxide levels might provide useful diagnostic and prognostic information about disease. however, larger studies involving more patients are needed.

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