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Possible Diagnostic/Prognostic Role Of Survivin And MMP3 In Breast Cancer Disease

Sohair Aly Hassan[1*], **Olfat Hammam**[2], **Than Than Htwe** [3]& **Hassan Zaki Ghanem**[1]
[1*] Therapeutical chemistry Dept /National Research Center, Cairo/ Egypt &Ibnsina College for Medical Studies,KSA
[1]Therapeutical Chemistry Dept/National Research Center, Cairo/Egypt
[2]Theodor Bilharz Research Institute (Patholology Dept) ,Cairo/ Egypt
[3]Faculty of Medicine (Pathology Dept), Quest International University Perak (QIUP), Malaysia
Correspondence author: sohaireslamaya@hotmail.com

ABSTRACT—

The identification of new biomarkers may facilitate development of a novel serum-based assay that would permit early detection of breast cancer. Objective: This study was conducted to investigate the serum matrix metalloproteinase-3 (MMP-3) and survivin levels in patients with breast cancer and their relationship with known clinicopathological variables. Materials & methods: The study included 40 breast cancer patients, age ranged from 28-70 [mean age 54 yrs]. Serum samples were obtained 3 month post-surgery 20 of them were invasive ductal carcinoma & 20 were invasive lobular carcinoma, and 10 samples were collected from healthy people as control cases. The concentrations of MMP3 and survivin were determined by sandwich enzyme linked immuno-sorbent assay (ELISA). Results The best cutoff points for each of survivin and MMP3 were depicted by (ROC) curve. The mean and median levels of MMP3 showed significant values compared to controls ($p < 0.001$) & ($p < 0.000$). Serum MMP3 was significantly correlated with high grades and stage ($p < 0.001$). Each of survivin and MMP3 achieved the highest sensitivity level 100% followed by positive predictive values 90% toward invasive lobular carcinoma type. Conclusion: Survivin and MMP3 is a statistically significant diagnostic / prognostic marker in blood serum for breast cancer detection in general and might be new markers for the detection of invasive lobular carcinoma type in particular.

Keywords—MMP3, survivin,ductal carcinoma, lymph node, stage & grade:

INTRODUCTION

Breast cancer represents the most commonly diagnosed cancer in women and the second leading[1], [2], [3], [4] and [5]. In Egypt, breast cancer is the most common cancer among women, representing 18.9% of total cancer cases (35.1% in women and 2.2% in men) [6]. Breast cancer in Egyptian patients has a younger age distribution with majority of cases occurring between 30–60 years of age. The most common type of tumor was invasive duct carcinoma (83.4%), while intraductal carcinoma was present in 1.5% of cases. Invasive lobular, medullary and mucoid carcinoma were detected in 7.1%, 1.6% and 2.3% of cases respectively. Pathologic grading showed a low incidence of grade I (5.4%). Grades II and III tumours were 66.0% and 28.6% respectively [7]. This fact emphasizes the importance of selecting sensitive diagnostic and prognostic markers in the early stage and more efficient targeted treatment for this disease. The presence of circulating tumor cells may be of much greater importance in the management of patients with early stages I – III breast cancer [8].

Survivin is a cancer gene that is silenced in differentiated tissues, while overexpressed at high levels in vast majority of tumors. It has garnered great interests in recent years. Some essential properties characterizing it as an ideal target as it involve inhibiting apoptosis, promoting mitosis, [9], [10] and [11]. Stimulating vessel growth thus inducing chemo-resistance [12], [13]. These functions touch the full gumat of tumor genesis, including proliferation, migration, invasion, and collectively facilitate malignant behavior. In the case of breast cancer, survivin detection independent or combined in serum and/or urine has emerged as a measure for diagnosis. Moreover, many studies indicated that aberrant expression of survivin is associated with poor prognosis and drug or radiation resistance. Strategies targeting survivin to treat breast cancer have got promising initial results.

Matrix metalloproteinase's (MMPs) are a family of more than over 20 proteins , ubiquitous in tissues and biological fluids and are produced by either tumor cells or host peri-tumoral cells It is believed that their profile in the blood could serve as biological markers for disease on- set, progression and monitoring [14],[15]. MMPs also share Survivin on the same ground, since it can stimulate, increase proliferation, resistance to apoptosis (anti-apoptotic), and can be considered as one of the checkpoint(s) in regulating cell death and activation of growth factors and growth factor receptors [16], [17], [18], [19], [20], [21], [22], [23]. Moreover, MMP3 also known as

STR1, STMY1 (stromelysin-1), [24] and [25] plays a key role in the tumor growth, as it degrades interstitial type I and III collagens and many extracellular matrix proteins and cell-surface molecules, including collagens, tumor necrosis factor-α precursor, and E-cadherin [26], and activate other MMPs [27]. Furthermore, it can induce molecular events leading to epithelial-mesenchymal conversion and spontaneous premalignant lesions in mammary glands of transgenic mice [26]. Over expression of survivin and MMP3 by cancer cells may lead to anti-survivin, anti-MMP3 antibody responses and cytotoxic T-lymphocyte responses against the cancer [28] and [29].

In the present study, we examined the occurrence of circulating antibody response against survivin & MMP3 as a prognostic and predictive markers in relation to histopathologic finding in patients with breast cancer of both types lobular & ductal carcinoma.

Exclusion criteria and Inclusion :

Criteria for exclusion from the study included distant metastasis at the time of diagnosis. Severe renal, hematological, hepatic and cardiac dysfunction and any disease can affect the study measurements. Tumor samples and clinical information.The study was performed in conformity with declaration of Helsinki II and was approved by the ethical review committee of the National Research Center (NRC). All patients included gave written informed consent to participation in the study.

MATERIAL & METHOD

Forty cases with primary breast cancer with age ranges 28--70 were selected for the present study. Basic blood investigations, chest x-ray, ECG and CT scan were done for all the patients and the diagnosis was confirmed. Core needle biopsy was done. Patients were then assessed according to the pathological TMN classification [30].The patients were then subjected to surgery with or without neo-adjuvant chemotherapy. Blood samples were drawn using standard phlebotomy procedures without anticoagulant for the determination of Survivin and MMP3 levels after 3 month post-surgery. Blood was allowed to coagulate for up to 2 hours at room temperature. Sera were separated by centrifugation 1500 g for 20 min at 4°C., immediately aliquoted, frozen, and stored at -80°C. No more than two freeze–thaw cycles were allowed for any sample .The clinical features for the study group were 21 patients with low grade II, low stage (T2b) and the remaining (n = 19) were with high grade tumor III, high stage (T3b). Lymph node positive involvements were detected in 28 patients, hormonal receptors were classified as 7 cases with ER-positive and 33 with PgR-positive, HER-2/neu was detected in 28 breast cancer patients. The control group consisted of 10 normal, healthy women (mean age 35 ± 13, range 17–67).

Measurement of MMP3:

Serum levels of MMP-3, was measured using a Biosource International Inc Gamarillo, California USA, as previously described [31]. All samples were assayed in duplicate. According to the manufacture's protocol, 100 µl of diluted serum samples were added to the assay and the levels of MMP-3, was determined using appropriate peroxides'-conjugated anti- MMP-3, antibodies. The reactions were stopped by the addition of 100 µl of 1 M sulfuric acid and absorbance of the product was read at 450 nm within 30 minutes.

Measurement of Survivin:

Total human serum survivin concentrations were analysed by an enzyme-linked immunosorbent assay (ElISA) method using commercial reagents [Titer zyme EIA;Assay {Biosource International Inc Gamarillo, California USA.

Statistical analysis:

Statistical analysis was performed using SPSS version 11.0 (SPSS, Chicago, IL).Quantitative data were statistically represented in terms mean standard division (SD) and median (Table 2). Comparison between the presented groups in this study was done using Independent samples T-Test for comparing two parametric groups, and using Mann-Whitney Test for comparing two nonparametric groups, and Kruskall-Wallis Test was used when comparison between more than two nonparametric groups.The ROC-Curve method was used to generate the Cut-off value [32], Area under the curve, Sensitivity (the ratio of patients with breast cancer who were positive for the variable), Specificity, (the ratio of patients without breast cancer who were negative for the variable) Positive Predictive Value, Negative Predictive Value and Test Efficiency for Survivin and MMP3 were measured. $p < 0.05$ was set to be level of significance .

RESULTS

A series of 40 female patients with unilateral, resectable breast cancer were included in this study. The mean ± SD age of the patients was 54 ± 3.2 years. Ten age-matched healthy female volunteers were recruited as controls. The clinical features of the breast cancer patients are outlined in (Table 1). Out of them 20 were invasive duct carcinoma and the remaining (n = 20) were invasive lobular carcinoma. Levels of serum survivin and MMP3 in patients with breast cancer and in healthy controls are shown in (Table 2). There were a marked significant difference in serum mean levels in both of survivin, MMP3 between breast cancer patients and control group. Tumor grade and stage have shown a highly marked significance in relation to the levels recorded for MMP3 only. As shown in (table 2). None of the prognostic parameters analyzed correlated significantly with serum survivin. Receiver operating characteristic (ROC), (Figure 1 A, B) depicts the survivin and mmp3 ROC curves. A ROC analysis estimates a curve, which describes the inherent trade-off between the sensitivity and specificity of a diagnostic test. Each point on the ROC curve is associated with a specific diagnostic criterion. The area under the ROC curve (AUC) may be regarded as a mean of the sensitivity of all possible specificities. The diagnostic measure with the higher AUC is typically regarded as better. Thus Survivin (AUC = 0.840) , MMp3(AUC = 0.700) had a good accuracy of all possible cutoffs in cases of lobular carcinoma. The cutoff ,109.95 ng/mL&5.71 ng/ml of survivin and MMp3 respectively in serum yielded a sensitivity of 100 % and a specificity of 80% for each of survivin and MMP3 respectively in patients of invasive lobular when considering breast cancer as true-positive cases and all non–breast cancer subjects as true-negative cases (Compared with their specificity and sensitivity in case of invasive ductal ,survivin was higher than MMP3 95% , 80

%, suggesting that survivin together with MMP3 could be an option for the clinical prognosis of breast cancer of ductal type.

***Table 1*:** disease characteristics of the primary breast cancer patients (n = 50)

Groups		N
Pathological characteristics		
Breast cancer	Control	10
	Invasive duct ca	20
	Invasive lobular ca	20
Tumor size	≤ 2 cm—T2 ≥ ± 5 cm or T3	21 19
Lymph node involvement	Negative	12
	Present	28
Tumor Grade	II	21
	III	19
Tumor Stage	T2b	21
	T3b	19
Estrogen receptor ≥ 10 fmol/mg	Negative	33
	Positive	7
Progesterone receptor ≥ 10 fmol/mg	Negative	33
	Positive > 10 fmol/mg	7
Adjuvant chemotherapy	No	33
	Present	7
HER-2/neu expression Negative Positive	High ≥15ng/mL	12
	Low≥15ng/mL	28
Age, mean (range 28-70 years)	Mean 37.8 years	

Hormone receptor status: negative, estrogen and progesterone receptor (<10 fmol/mg protein); positive, estrogen and/or progesterone receptor (±10 fmol/mg protein).HER2 status, negative 15 ng/mL/mg protein; positive ±15 ng/mL protein.

***Table 2*:**

Tumor Markers			Survivin Pg/Ml		MMP3 Pg/ML	
	Groups	N	Mean ± SD (Median)	P value	Mean ± SD (Median)	P value
Breast Cancer	Control	10	94.93 ± 15.02 (94.25)	0.000*	4.48 ± 1.23 (3.98)	0.001*
	Invasive duct ca	20	195.59 ± 51.73 (202.92)		11.02 ± 7.72 (10.60)	
	Invasive lobular ca	20	254.28 ± 71.86 (221.39)		13.76 ± 4.83 (13.55)	
Lymph node involvement	Negative	12	236.86 ± 74.14 (218.31)	0.525	14.34 ± 6.51 (17.95)	0.147
	Present	28	219.82 ± 66.81 (216.80)		11.55 ± 6.44 (9.15)	
Tumor Grade	II	21	214.35 ± 83.04 (212.74)	0.207	6.86 ± 3.13 (8.99)	0.000*
	III	19	236.63 ± 47.46 (216.80)		18.50 ± 2.40 (18.95)	
Tumor Stage	T2	21	214.35 ± 83.04 (212.74)	0.207	6.86 ± 3.13 (8.99)	0.000*
	T3	19	236.63 ± 47.46 (216.80)		18.50 ± 2.40 (18.95)	
Estrogen receptor	Negative	33	221.21 ± 69.39 (212.74)	0.277	11.85 ± 6.65 (9.46)	0.205
	Positive	7	242.48 ± 66.73 (216.80)		14.95 ± 5.48 (18.95)	
Progesterone receptor	Negative	33	221.21 ± 69.39 (212.74)	0.277	11.85 ± 6.65 (9.46)	0.205
	Positive	7	242.48 ± 66.73 (216.80)		14.95 ± 5.48 (18.95)	
Chemotherapy	No	33	221.21 ± 69.39 (212.74)	0.277	11.85 ± 6.65 (9.46)	0.205
	Present	7	242.48 ± 66.73 (216.80)		14.95 ± 5.48 (18.95)	

Survivin pg/mL & MMp3 pg/mL in primary breast cancer from both types in correlation with histological characteristics. P<0.05 is the level of significance.

***Table 3*:**

	Survivin		MMP3	
	Invasive ductal carcinoma	Invasive lobular carcinoma	Invasive ductal carcinoma	Invasive lobular carcinoma
Cut-off value	109.95		5.71	
Area under the curve	0.487	0.840	0.537	0.700
Sensitivity	95.0%	100.0%	55.0%	100.0%
Specificity	80.0%	80.0%	80.0%	80.0%
Positive Predictive Value	90.5%	90.0%	84.6%	90.0%
Negative Predictive Value	88.9%	100.0%	47.1%	100.0%
Test Efficiency	90.0%	93.3%	63.3%	93.3%

Table 3 shows area under the curve, survivin & MMP3 was represented by the highest area under the curve 0.840 followed by 0.700 respectively in case of lobular carcinoma. Survivin and MMP3 demonestrated equal values for sensitivity in case of Invasive lobular carcinoma and equal specificity almost for each of invasive lobular and ductal carcinoma . positive, negative predictive values were high 90 % in each of survivin and MMP3 .

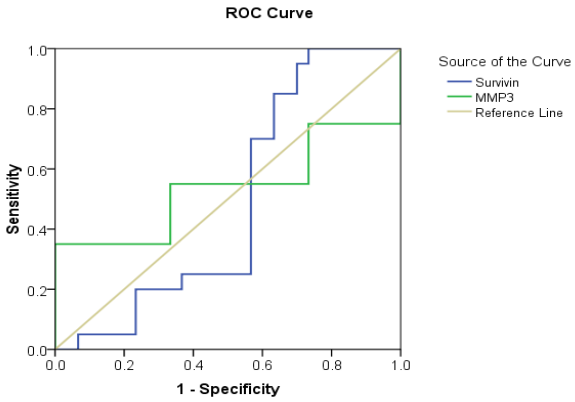

Figure 1 A: Invasive ductal carcinoma group

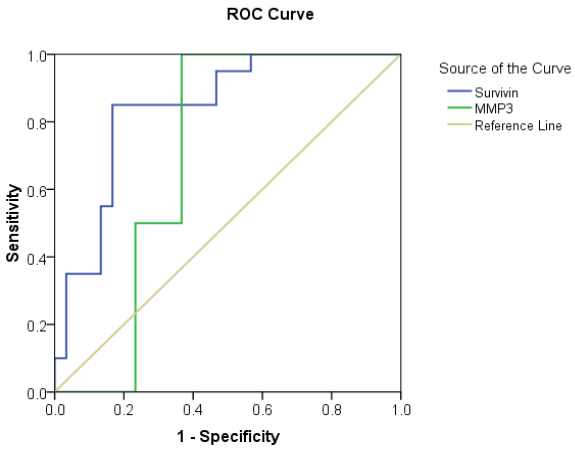

Figure 2 B

Figure1A& B: a Receiver operator characteristic curves for diagnosis breast cancer from both types versus noncancerous cases. Curves demonstrate the relative accuracy for the individual serum levels of both survivin and MMp3 to discriminate between breast cancer and control cases. Serum levels of healthy control subjects (n = 10) were considered true-negative cases, whereas serum levels of patients with confirmed breast cancer (n = 40) were considered true-positive cases. The AUC was 0.0.84 (95% confidence interval for survivin, 0.700 (95% confidence)for MMP3 At a cut-off value 109.95 ng/mLfor survivin and 5.71 ng/ml for MMP3

DISCUSSION

Breast cancer has been posing a great challenge with an overall poor long-term prognosis [33]. Since survivin and MMp3 are selectively expressed in malignant tissues, and can inhibit apoptosis, promote cell division and enhance angiogenesis [34], its detection in body fluids could serve as an ideal tumor markers for prognosis and prediction.
In this study, survivin & MMP3 expression, as prognostic & predictive markers were measured by ELISA, in patients with breast cancer of both types, invasive ductal & lobular carcinomas [stage 11-111]. Initially the levels of Survivin and MMP3 expression were noticed to be significantly increased in breast cancer patients as compared to control [Table,2]. These results were in full consistence with [35] ,[36] and [11] who detected survivin expressing circulating breast cancer cells in the peripheral blood as well as survivin with its protein variants in breast cancer patients, but not in the healthy controls. However positive nodes and the histological grade, always form a predictive index that may be an excellent and simple guide for the clinical decision of subsequent therapy. In the present study, Survivin showed marked increments with the indices of high grade ,high stage, positive nodal metastases, hormonal status receptor ,HER-2, and chemotherapy although a lack of significance. These results have been confirmed before by [35] and [37] as they observed a significant association between the survivin expressing circulating breast cancer cells with various clinicopathological parameters and also with [38] ,[39] and [37],in a study investigated survivin in serum and urine using real-time PCR and Elisa techniques. The association of the survivin level in the present few 7 cases of chemotherapy and the 12 cases of negative lymph node could be an indicator for poor prognosis but also may be a good sign for a good response to chemotherapy to be in a full agreement with [33].

Recalling that ER/PR status represents the best predictive marker that is currently in use to determine which patients are most likely to benefit from endocrine therapy [40].In the present study 33 out of 40 patients were shown to be (–ve) estrogen while only 7 (+ve) cases recorded high mean level of survivin the matter which could be explained as a worth prognosis in both –ve and positive associated estrogen. This explanation could be supported by [41]& [42] who reported that increased survivin expression is more commonly seen in estrogen receptor negative carcinomas and is associated with a poor overall prognosis. moreover the results as illustrated in table (3) suggested that serum survivin could be a sensitive marker for detecting lobular breast cancer type with 100% sensitivity in consistence with a more recently, research from China reported that detection of survivin or other associated gene may serve as an important sensitive diagnostic test for breast cancer and provide an early biomarker of aggressive tumor behavior before the appearance of distant metastasis. However the authors believed the detected levels of MMP3 may be like other MMP2 and/or MMP9 in the blood to serve as biological markers for disease on- set, progression and monitoring [43] , [44] and [45].

On the other hand although available reports suggests MMP implications in the pathological expression in many tumors including breast cancer , but correlations in breast cancer are still at a nascent stage [46] and [47]. Such discrepancies could be due to different pre analytical ways of sample processing [48], for example, addition of some preservatives, eg EDTA, heparin or clot accelerator may affect the bio nature of the prepared sample in terms of inhibition ,or binding to MMPs in case of heparin or allow release for a negligible amounts of gelatinizes in the presence or absence of glot accelerators ,for this reason a naïve serum is a better option for measuring the concentration of circulating MMPs in patients [49].

Back to our results as illustrated in [Table, 2] a significant increase in serum MMP-3 level in the cancer patients when compared to control group($P < 0.001$). This finding

correlates with the assumption that the concentration of these gelatinases is expected to be high because of their distinct role in cancer associated tissue remodeling indicating that signals from the tumor cells that is; soluble factors, or direct cell contact activate the production of these enzymes in the surrounding cells/tissue. These signal increased the production of MMP3, which degrades the extracellular matrix and aids angiogenesis. Moreover these results confirmed by a study done by[2] as he reported the gradually post-surgery increment in MMP3 level as an evidence for recurrence. Our data revealed a strong involvement of mmp3 in the ductal as well as the lobular type (Table, 2),which can be interpreted based on the strong association and implication of mmp3 in the branching of the breast ductal type as a result of depolarization of epithelial cells when initiating new branches [50] , [51], [52], [53] and [54].In fact the results of the two markers studied here may give evidence to be good prognostic ones since they showed increment only after 3 month post primary surgery, the matter which can be in full agreement with [55] and [26] since they recorded a gradual increment of plasma MMP-9 activity 1 to 8 months before the clinical diagnosis of recurrence in all patients who suffered a relapse of disease. An important feature of the tumoral invasive phenotype is the capability to produce lytic enzymes, such as MMPs; therefore, an increase in these enzymes may correlate with a more advanced and invasive tumor [2].Our results interestingly supported this theory. MMP3 showed a marked significant strong correlation with, high tumor grade, and high stage 0.001, 0.000 respectively. But not with the lymph node metastases, chemotherapy or hormone receptor status Her-2 (Table 2)., So our data seem to be in accordance with[56].[57]; [58];[59]; [60];[46], [61] and[18] . Moreover, our results seem to be compatible with a study done by[62],as he recorded that MMP3 serum biomarker levels from breast patients could distinguish between clinical response groups with 82% sensitivity and 73% specificity, but the only difference that we used mmp3 as a marker after primary surgery so it could be a good evidence for early detection of recurrence. Moreover It could be easily seen that most of the significant satisfactory values of sensitivity and specificity were represented by survivin and MMP3 in the lobular carcinoma type 100%, 80% respectively (Table,3).Our results likely to be close to that of [63] in a study investigating survivin sensitivity in the serum of retinoplastoma patients . These results could conferred significant power to conclude that each of survivin and MMP3 could serve as a sensitive prognostic tool for the early detection of the recurrence of the disease after the primary surgery.

CONCLUSION

The study results demonstrated that serum biomarkers [Survivin, MMP3] can deliver improved clinical value, and can classify or predict breast cancer patients at high risk with specific type of recurrence after primary operation. Further investigations based upon this initial study are highly required.

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Competing interests

The authors declare that they have no competing interests.

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