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Combining nuclear matrix protein-52, collagen III and matrix metalloproteinase-1 for more effective breast cancer early detection

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Running title: NMP, collagen III and MMP-1 for BrCa

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Abstract

Aim: Improvement breast cancer (BrCa) control will be markedly supported by early detection. Owing to limitations of current diagnostic tools like mammography and ultrasound and lack of existing confirmed BrCa biomarkers, this study concerned the evaluation of some potential biomarkers and their combination in BrCa detection. **Methods:** Three hundred participant women; 200 with BrCa patients, 50 with benign breast diseases and 50 healthy individuals were enrolled in this study. Serum levels of nuclear matrix protein-52 (NMP-52), collagen III and matrix metalloproteinase-1 (MMP-1) were determined by ELISA. **Results:** Mean levels of NMP-52 (9.83±1.1 μg/ml), collagen III (22.6±3.2 μg/mL) and MMP-1 (3.6±0.3 μg/mL) in BrCa patients were significantly higher (P<0.0001) than benign (5.8±0.7, 12.2±1.3 and 2.6±0.23 μg/mL, respectively) and healthy (1.2±0.1, 6.0±0.2 and 1.66±0.04 μg/mL, respectively) groups. Also, these levels were associated with the tumor progression and may reflect the BrCa disease severity, high serum levels of these markers have been associated with tumor advanced stages (T3-T4), high grade (G3), and large size (>2cm). Diagnostic score combined these markers revealed valuable power (AUC=0.83, 78% sensitivity, 75% specificity) in BrCa diagnosis. This power not markedly influenced in detection of early tumor stages (T_{is}-T2), low grade (G1-G2), lesser tumor size ≤2 cm and negative lymph nodes status (AUC=0.79, 0.74, 0.74, and 0.85, respectively).**Conclusions:** Combined use of NMP-52, collagen III and MMP-1 can serve as potential biomarker for BrCa diagnosis. This combination is likely to improve the clinical early tumor diagnosis.

Keywords: Breast cancer, Collagen, Matrix metalloproteinase, Nuclear matrix protein, Score

1 INTRODUCTION

BrCa is the most incidence cancer among female, almost 1.7 million BrCa cases are diagnosed worldwide. It is accounting for 15% of all women mortality **(Torre et al., 2015)**. BrCa early detection, to ameliorate its survival and outcome, remains the backbone of the disease monitoring **(Nie et al., 2018)** Presently, BrCa diagnosis depends mostly on mammography. Although, mammography screening has some limitations (low sensitivity and specificity) **(Pace and Keating, 2014; Welch et al., 2016)**. Otherwise, blood tumor marker is more acceptable and could also overcome imaging limitations **(Kazarian et al., 2017)**. So that, there is an urgent need to develop a biomarker for breast cancer early detection **(Nie et al., 2018)**. Morphological modification in cell nucleus is considered one of the first malignant transformation signs. The nuclear matrix is the nucleus structural framework which is believed to be participated in nuclear morphology, DNA replication and organization, nuclear regulation, stress responses, and RNA synthesis **(Choi et al., 2014)**. Alterations or aberrant expression of specific nuclear matrix proteins (NMPs) have been linked with progression of malignant **(Choi et al., 2014)**. NMPs are involved in BrCa tumor progression and malignant transformation **(Sjakste et al., 2004)**. Cancerous cells release NMPs into the bloodstream, thus the detection of NMPs in cancer patients might be serve as a good candidates for tumor markers **(Luftner and Possinger, 2002)**.

On the other hand extracellular matrix (ECM) is a main component of tumor stroma that is accounted for regulation of tissue and cell functions. ECM is a critical source for motility, angiogenic, growth, and survival factors that affect tumor progression and biology **(Lai et al., 2011)**. Collagens have been regarded the main proteinaceous components of the ECM. In BrCa, the immunohistochemical expression of collagen was positively correlated with the size of tumor and inversely with other prognostic factors including estrogen and progesterone receptors **(Ioachim et al., 2002)**. Furthermore, ECM is not a static structure but it is remodeled constantly by proteolytic enzymes like the matrix metalloproteinases (MMP) or through the cells by lysosomal enzymes **(Gialeli et al., 2011)**. MMPs play an important role in metastasis and invasion of

BrCa. MMPs mRNA expressions were upregulated in BrCa tissues **(Benson et al., 2013)**. Thus, in this study we aimed to evaluate the serum levels of NMP-52, collagen III and MMP-1 in patients with breast diseases and to investigate the potential value of a novel diagnostic score developed from these markers for BrCa early detection.

2 METHODS

2.1 Patients

The study enrolled 300 participants as follow: breast cancer female patients (n=200), benign breast disease female patients (n=50) and healthy female individuals (n=50). They recruited from Oncology Center, Mansoura, Egypt. Diagnosis of breast cancer was pathologically confirmed. None of women with benign diseases or the normal women had a record of any cancer. Patients' data regarding demographic, lymph node status, tumor size, tumor grade and clinical staging of disease were collected from the histopathology reports. The study has been performed according to the ethical guidelines of Helsinki Declaration.

2.2 Samples and laboratory assays

Blood samples were obtained and permitted to clot at room temperature for (20-30) minutes. The blood samples were centrifuged, sera were stored at -20°C until being analyzed. The levels of NMP-52, collagen III and MMP-1 were analyzed using ELISA. The measurements were made according to Attallah et al protocols for NMP-52 **(Attallah et al., 2015)** , collagen III **(Attallah et al., 2007)** and MMP-1**(Attallah et al., 2011)**.

2.3 Statistical analysis

The SPSS software and GraphPad Prism were used for all data analysis. Data were presented as percentage and mean± standard deviation (SD). Chi square, ANOVA, student *t*-test or Mann–Whitney U-test was used to compare between studied groups. Stepwise linear regression analysis was used to develop a novel score. Simplified score was calculated by summing up the single markers. Area under the curve (AUC) was used for evaluating the diagnostic performances of single and combined markers. Sensitivity and specificity were determined from a 2×2 contingency table, *P* value less than 0.05 was considered as a statistically significant value.

3 RESULTS

3.1 High circulating levels of candidate markers were associated with BrCa progression

The baseline clinicopathological characteristics of patients and healthy individuals enrolled in this study is presented in Table 1. Mean NMP-52 level was significantly (P<0.0001) elevated in BrCa (9.8±1.1 µg/mL) compared with benign (5.8±0.7 µg/ml) and healthy (1.2±0.1 µg/mL) controls (Figure 1A). Also, BrCa was associated with high (P<0.0001) levels of collagen III (22.6±3.2 µg/mL *vs.* 12.2±1.3 for benign and 6.1±0.2 µg/mL for healthy; Figure 1B) and MMP-1 (3.6±0.3 µg/mL *vs.* 2.6±0.2 µg/mL for benign and 1.7±0.1 µg/mL for healthy; Figure 1C). Regarding histopathological features, elevated serum levels of NMP-52, collagen III and MMP-1 were associated with advanced cancer stages, high tumor grade, large tumor size and positive lymph node metastasis and so it may reflect the BrCa severity (Table 2).

3.2 Development and diagnostic performances of BrCa diagnostic score

Combination of candidate markers, as diagnostic score, revealed values that were significantly (P<0.0001) elevated in BrCa (35.9±3.6) compared with benign (20.5±1.3) and healthy (8.9±0.3) controls (Figure 1D). Moreover, these elevated values were associated with tumor late stages, high grade, large size and positive nodal status (Table 2). Using ROC curve analysis, score values yielded AUC of 0.83 when differentiate all BrCa patients from all non-cancerous (healthy individuals and benign patients combined) with 78% sensitivity and 75% specificity. Diagnostic performances of score were greater than single marker (Table 3). Score AUC rose to 0.94, when patients with only BrCa late stages compared to all non-cancerous

with 100% sensitivity and 75% specificity. This power not markedly influenced in detection of early tumor stages (T_{is}-T2), low grade (G1-G2), lesser tumor size ≤2 cm and negative lymph nodes status (AUC=0.79, 0.74, 0.74, and 0.85, respectively); Figure 2.

TABLE 1 Clinicopathological characteristics of the study populations

Clinicopathological features	Value
Healthy individuals	
No. (%)	50 (16.7)
Mean age ±SD, years	48.3 ± 15.7
Benign breast disease patients	
No. (%)	50 (16.7)
Mean age ±SD, years	49.5±11.5
Fibroadenoma, no. (%)	45 (90)
Hamartoma, no. (%)	5 (10)
Cancer patients	
No. (%)	200 (66.6)
Mean age ±SD, years	50.4±12.1
Histopathological type, no. (%)	
ductal carcinomas	161 (80.5)
lobular carcinomas	24 (12)
Others	15 (7.5)
Lymph nodes involved, no. (%)	
Negative	33 (16.5)
Positive	141 (70.5)
Unknown	26 (13)
Distant metastases, no. (%)	
Negative (M_0)	145 (72.5)
Positive (M_1)	11 (5.5)
Unknown	44 (22)
T stage, no. (%)	
early stage (T_{is} - T2)	131 (65.5)
late stage (T3 – T4)	69 (34.5)
Histological grade, no. (%)	
low grade (G1-G2)	115 (57.5)
high grade (G3)	85 (42.5)
Tumor size, no. (%)	
≤ 2 cm	54 (27)
>2 cm	106 (53)
Unknown	40 (20)

TABLE 2 Levels of candidates and combined markers according to tumor severity features

	NMP-52 (µg/mL)	CollagenIII (µg/mL)	MMP-1 (µg/mL)	Score
Tumor stage				
Early stage (T_{is}-T2)	7.1±1.7	14.9±3.1	3.2±0.7	25.5±4.5
Late stage (T3–T4)	16.1±5.3	38.6±10.7	4.7±1.1	59.5±13.9
**P value*	<0.0001	0.002	0.02	<0.0001
Tumor grade				
Low grade (G1-G2)	7.9±1.9	13.1±2.9	2.4±0.4	24.1±3.4
High grade (G3)	13.2±3.8	22.46±6.5	3.4±0.8	33.9±8.5
**P value*	0.03	0.002	0.01	0.001
Tumor size				
≤ 2 cm	6.7±1.1	13.6±3.3	2.4±0.3	22.9±2.9
>2 cm	11.8±2.5	25.6±7.1	4.1±0.7	41.8±8.0
**P value*	0.04	0.1	0.01	0.02
Lymph nodes involved				
Negative	6.1±1.5	20.4±4.9	2.6±0.4	30.8±5.2
Positive	10.9±2.5	24.8±6.6	3.8±0.8	38.4±5.9
**P value*	0.1	0.6	0.5	0.3

Variables were expressed as mean ± SD. NMP-52= nuclear matrix protein-52, MMP-1= Matrix metalloproteinases-1, Score=NMP-52+Collagen III+MMP-1. **P*<0.05 is considered significant.

TABLE 3 Diagnostic performances of single and combined markers for breast cancer diagnosis

Parameter	Cut off	AUC	Sensitivity	Specificity
Healthy *vs* breast cancer				
NMP-52 (µg/mL)	7.8	0.92	54	100
Collagen III (µg/mL)	11	0.95	60	100
MMP-1 (µg/mL)	2	0.87	62	100
Score	20	0.99	78	100
Non-cancerous *vs* breast cancer				
NMP-52 (µg/mL)	7.8	0.75	54	75
Collagen III (µg/mL)	11	0.79	60	75
MMP-1 (µg/mL)	2	0.70	62	63
Score	20	0.83	78	75
Benign *vs* breast cancer				
NMP-52 (µg/mL)	7.8	0.64	54	60
Collagen III (µg/mL)	11	0.64	60	60
MMP-1 (µg/mL)	2	0.60	62	50
Score	20	0.73	78	60

AUC= area under receiver-operating characteristic curve, NMP-52= nuclear matrix protein-52, MMP-1= Matrix metalloproteinases-1, Score=NMP-52+ Collagen III+ MMP-1.

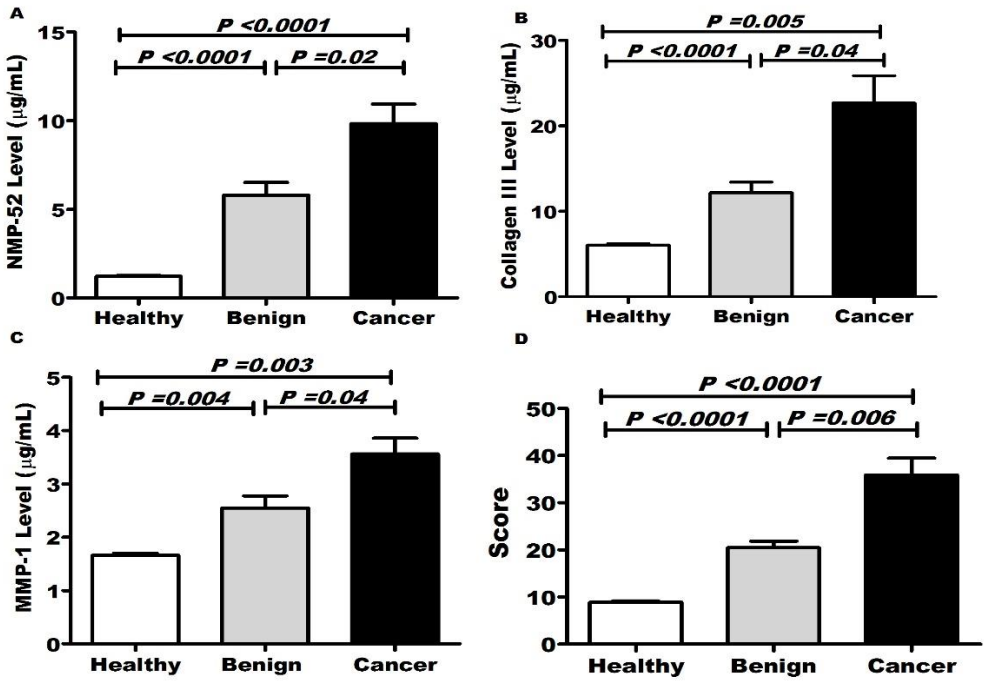

FIGURE 1 Levels of NMP-52 (**A**), collagen III (**B**), MMP-1 (**C**), developed score (**D**) in healthy, benign and cancer groups. The highest significant difference between benign and cancer groups obtained by the developed score. Score=NMP-52+ Collagen III+ MMP-1. *P*<0.05 is considered significant.

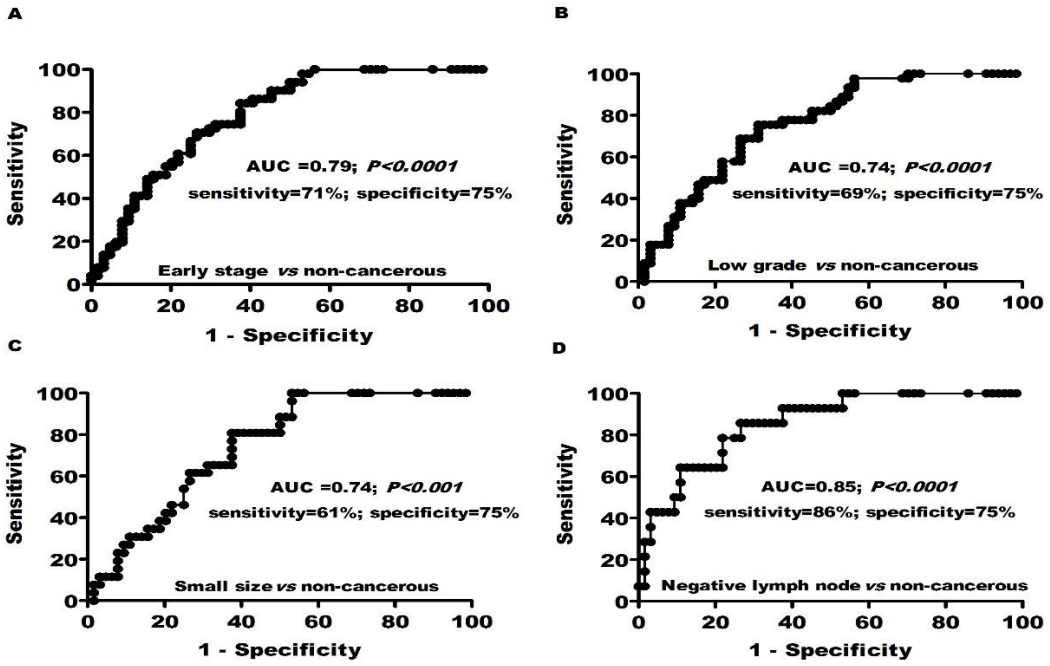

FIGURE 2 ROC analysis for the developing score. (**A**) To discriminate patients with early BrCa stages from all non-cancerous individuals. (**B**): To discriminate patients with low BrCa grade from all non-cancerous individuals. (**C**): To discriminate patients with small size from all non-cancerous individuals. (**D**): To discriminate patients with negative lymph node from all non-cancerous individuals. Score= NMP-52+ Collagen III+ MMP-1. *P*<0.05 is considered significant.

4 DISCUSSION

Efficient biomarker for breast cancer diagnosis could be a beneficial and less invasive than other pathological tests **(Chung and Baxter, 2012)**. Cancer clinical diagnosis is based, in part, on pathognomonic changes including nuclear irregularity and enlargement, and altered chromatin organization. Nuclear morphology as well as gene expression is partially controlled by the nuclear matrix including NMP **(Luftner and Possinger, 2002)**. Definite changes in NMP composition and chromatin structure involved in breast tumor progression **(Barboro et al., 2012)**. NMP recognizes human epidermal growth factor receptor-2 and enhance its expression. The HER2 gene overexpression has a major role in BrCa pathogenesis. NMP protein is expressed with HER2 in breast cancer but absent in normal tissues **(Sjakste et al., 2004)**. NMPs are involved in breast tumor progression. Here, by studying the NMP-52 serum level, we found that NMP-52 level was significantly higher in BrCa patients than benign and healthy groups. Moreover, this level is associated with tumor progression and advanced cancer histopathological parameters. During cancer the ECM controlled homeostasis is disturbed as well as stiffens of ECM and changes in protein composition are occurred, and increased levels of proteases are secreted **(Bager et al., 2015)**. These processes are resulted in secretion of collagens to the blood that when analyzed can reflect progression of disease **(Bager et al., 2015)**.

Therefore, it was appear that collagen play a key role in regulating BrCa progression. Increased collagen alters cellular morphology to a more proliferative and invasive phenotype **(Maskarinec et al., 2013; Provenzano et al., 2009)**. We found that collagen III level may reflect the BrCa disease severity as high collagen III serum concentration has been associated with aggressive tumor features. Bager et al. found that increased levels of collagen III were elevated with progression of BrCa **(Bager et al., 2015)**. These elevated levels can be owing to increased degradation resulting from the invasion process or can be an index of high level of angiogenesis **(Hewitt et al., 1992)**. From another hand, some studies have elucidated a positive engagement between MMP levels and cancer metastatic of lung, colon, breast, head and neck, basal cell, ovarian, prostate, thyroid and gastric carcinomas **(Sunami et al., 2000)**. For instance, MMP-1 expression associated with poor prognosis in oesophageal and colorectal cancer **(Murray et al., 1998; Murray et al., 1996)**. MMPs can promote tumor growth by enhancing angiogenesis and by degrading matrix barriers **(Duffy et al., 2000)**. They can promote tumor growth and invasion by generating α1-antitrypsin cleavage product. They can also change cell cycle checkpoint control and allow genomic instability through affecting cell adhesion **(Sternlicht et al., 2000)**. MMPs can degrade all ECM ingredient which directly determine the synthesis and deposition rates of collagen in all tissues **(Gialeli et al., 2011)**. Although, it is well known that, collagen III distribution is inversely paralleled to MMP-1expression. We found that both circulating levels of collagen III and MMP-1 were elevated and these levels were associated with BrCa severity. Beside the traditional role of collagen as an inefficient obstruction to resist tumor cells, there is new evidence that it involved in promoting tumor growth **(Fang et al., 2014)**. In coordinated reciprocally processes, both decrease and increase collagen are participated in tumor progression **(Fang et al., 2014)**. Also, in breast cancer, MMPs are correlated with lysyl oxidase (LOX) expression **(Erler et al., 2009)** that increase MMPs levels which increase hydrolysis of collagen to reveal active sites producing a pro-tumorigenic environment to favor tumor progression **(Fang et al., 2014)**. Due to the tumor complexity, single tumor marker not have the sufficient ability to detect cancer tumors. Thus, combined markers improve the diagnosis **(Li et al., 2013)**. The combination of NMP-52, collagen III and MMP-1 is likely to improve the clinical tumor diagnostic sensitivity (78%). Moreover, our developed score has a sensitivity of 71% and specificity of 75% for BrCa early detection. These findings are well comparable to other single and combined markers for breast cancer diagnosis. The markers like

carcinoembryonic antigen (CEA), CA27.29 and CA15-3 have been indicated as diagnostic markers **(Kazarian et al., 2017)**. Our score performance is superior to these candidate markers for BrCa detection, the diagnostic sensitivities for CA 15-3, CA 27.29 and CEA were: (63%), (39%), and (22%), respectively **(Clinton et al., 2003)**. Differential epithelial membrane antigen and cytokeratin-1 ratio is similar to our score ability for BrCa early detection (sensitivity=72%, specificity=76%) **(Attallah et al., 2014)**. Also, our score diagnostic performance is slightly lower than Ławicki et al. results to assess the diagnostic utility of vascular endothelial growth factor (VEGF), and tissue inhibitors of metalloproteinase-2 (TIMP-2) in breast cancer early diagnosis. The combined use of VEGF and TIMP-2 with CA 15-3 resulted in the increase in sensitivity (83%), while with the combined use of the three parameters the sensitivity reaches to 93% **(Lawicki et al., 2017)**. Moreover, Zajkowska and Szmitkowski suggested that TIMP-2 and macrophage colony-stimulating factor are useful in BrCa diagnosis when combined with CA 15-3 (sensitivity= 84%; 78%, negative predictive value=71%; 65%, and AUC=0.89;0.87, respectively) **(Zajkowska and Szmitkowski, 2016)**.

CONCLUSION

The serum levels of NMP-52, collagen III and MMP-1 were potential biomarkers for diagnosis of breast cancer. Also, using NMP-52, collagen III and MMP-1 in combination represent a potential diagnostic marker. This combination produces a simple, accurate, cost effective and less invasive sampling assay that may be a valuable in breast cancer early diagnosis. Further large scale studies are needed to evaluate its performance in comparison to other established BrCa marker.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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