

Original Research Article

Caveolin-1 gene, hyaluronan and CA 15-3 as Potential biomarkers for diagnosis and prognosis of primary breast cancer

ABSTRACT

Aims: Breast cancer (BC) is the greatest female malignancy and the leading causes of cancer death in the less developed countries. The determination of markers that help in diagnosis, prognosis, discovery of recurrence and metastasis is a valuable tool for management in breast cancer patients. The present study aimed to provide new insights about the possible role of Caveolin-1 (Cav-1) gene, hyaluronan (HA) and cancer antigen (CA) 15-3 in development, progression and metastasis of breast cancer, evaluate the possible correlations between these biomarkers and the clinico-pathological status of breast cancer and compare between validity of these biomarkers with traditional breast tumor marker (CA 15-3).

Methodology: A total of 75 women whose ages ranged between 30 to 70 years and 10 healthy controls with matched age and sex were included. The patients were divided into 4 groups, group I: Included 39 female patients with breast cancer before operation, group II: Included 17 women from group I followed for 6 months after operation, group III: Included 9 women from group I followed for 12 months after operation, group IV: Included 10 female patients with benign breast diseases. Cav-1 gene analysis was performed by thermal cycler PCR method. Estimation of serum HA and CA 15-3 by ELISA and related clinico-pathological features were assessed.

Results: positive Cav-1 gene in 26 (66.7%) of 39 breast cancer patients was found. Benign and control groups were negative for the presence of the gene. Cav-1 gene was associated with larger tumor size ($p < 0.05$), grade ($p < 0.05$), advanced stage ($p < 0.01$) and lymphovascular invasion ($p < 0.05$). Caveolin-1 gene was detected in 5 of 17 cases after 6 months follow up. Twelve months after operation, no caveolin-1 gene was observed in the followed up patients. The mean serum levels of HA and CA 15-3 were significantly higher in breast cancer women before operation when compared to benign and control groups. Patients after 6 and 12 months follow up showed a decrement of CA 15-3 levels. Also, HA levels were changed towards normalization, 6 months after treatment.

Conclusion: presence of Cav-1 gene and high circulating HA, CA were significantly associated with breast carcinogenesis and metastasis. Accordingly, estimation of these biomarkers may expect the breast disease behavior and its prognosis.

Keywords: Biomarker, breast cancer, CA 15-3, cancer diagnosis, Cav-1 gene, hyaluronan.

1. INTRODUCTION

Breast cancer is the most common malignancy worldwide [1]. One in eight women in the United States will develop the illness in their lifetimes [2]. In Egypt, 37.7% of total cancer cases among women is breast cancer [3]. The addition of a blood-based tumor marker test may rise patient compliance as blood analysis is more acceptable [4].

Caveolin-1 is the principal structural constituent of caveolae micro domains, which represent a sub-compartment of the plasma membrane, elaborated in vesicular trafficking and signal transduction. Cav-1 gene maps to 7q31.1 and encodes a 21 to 24 kDa integral membrane protein. Caveolin-1 overexpression is associated with tumor malignant progression [5] and plays an important role in carcinogenesis because it interacts with many factors involved in mitogenic signaling, angiogenesis, and senescence processes. It is involved in breast cancer as a possible molecular target, able to predict the patient's response to treatment [6].

Hyaluronan (also hyaluronic acid or hyaluronate) is one of the most important polymers of extracellular matrix and could be affected with the physiological alterations in cell cycle during carcinogenesis. Hyaluronan binds

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mainly to CD44 receptor and promotes tumor growth, survival as well as cancer cell invasion [7]. Cancer antigen 15-3 is an epitope of a large transmembrane glycoprotein named mucin (MUC) that is resulting from the MUC1 gene. This protein is often overexpressed and aberrantly glycosylated on its extracellular region in breast cancer [8]. High tumor marker CA 15-3 before operation was significantly related with tumor size, axillary node involvement and advanced stage [9], so the present study clarify the possibility of using Cav-1 gene, HA, CA 15-3 as biomarkers for diagnosis and prognosis of breast cancer and correlate them with the clinicopathological status of BC.

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2. SUBJECTS AND METHODS

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2.1 Subjects

The current study included 75 female patients who were selected from the South Egypt Cancer Institute, Surgery Department, Assiut University, from December, 2013 to March, 2015. Their ages ranged between 30-70 years, with a mean $\pm$ SD (50.7 $\pm$ 10.3 years). In addition to 10 age and sex matched healthy controls. All participants signed a written informed consent for participation in the study. The study was approved by the Faculty of Medicine, Assiut University ethical committee in accordance with Helsinki declaration (1975).

Patients were divided into 4 groups as follows: Group I: Included 39 female patients with breast cancer before operation. Group II: Included 17 women from group I followed for 6 months after the surgical removal of their breast cancers. Group III: Included 9 women from group I followed for 12 months after the surgical removal of their breast cancers. Group IV: Included 10 female patients with benign breast diseases. Three pathological types of benign breast diseases were selected: 7 cases had fibroadenoma (70%), 2 cases had fibrocystic diseases (20%) and 1 case had phylloid tumor (10%).

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Group I women received treatment after surgery in the form of chemotherapy which included fluorouracil 500 mg/m², epirubicin 100 mg/m² or Adriamycin 50 mg/m² and cyclophosphamide 500 mg/m² (FEC or FAC) every 3 weeks for 6 cycles, then radiotherapy for 1.5 months followed by hormonal therapy (tamoxifen 20 mg/day) for patients whose tumors were positive for estrogen and/or progesterone receptors and to be continued for 3-5 years.

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The following was done for all participants including demographic characteristics, family history of breast cancer, smoking habits, therapeutic history and personal obstetric history. Imaging studies including mammography and ultrasound were done. Body mass index was calculated for each female.

Exclusion criteria included: the presence of any previous tumors, getting neoadjuvant chemotherapy or undergoing surgical operations for tumor resection, Patients with chronic medical diseases and patients who are unfit for surgery.

2.2 Laboratory Workup

Six ml venous blood was drawn by venipuncture, and divided into 2 parts. Two ml were collected on EDTA for DNA extraction, and the other 4 ml were left in room temperature for serum separation which was stored at -70°C till the assay of biochemical markers.

The level of serum hyaluronan was measured using an ELISA kit, supplied by WKEA Med Supplies (ChenguangGardon, China) according to the methods of [10]. Serum Cancer antigen 15-3 was measured using an ELISA kit, supplied by BioTina GmbH (Freiburg, Germany) according to [11].

Genomic DNA was isolated from peripheral whole blood collected on EDTA using QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. Primers were purchased from Invitrogen™ by life technologies, UK. The following primers were used to amplify 100 bp fragment of DNA.

Forward primer 5' AACGTTCTCACTCGCTCTCTGCTCGCTGCG 3',

Reverse primer 5' GTACACTTGCTTCTCGCTCAGCAC 3'

PCR was carried out using 25µl of Dream Taq PCR master mix (2x) (Sigma), 1 µg of template DNA, 12.5µl of nuclease-free water and 10µm of each forward and reverse primers. The amplification was conducted on ARKTIK 96 Thermal Cycler (thermoscientific) as follows: initial 5-min denaturation at 94°C, followed by 45 cycles denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 60s, then a final

95 extension step of 7 min at 72°C. PCR products were electrophoresed on ethidium bromide stained (0.5 µg/ml)
96 1% agarose gel containing 1X TEA buffer and visualized by TRZol UV transilluminator (USA).

97 2.3 Statistical Analysis

98 Data collected were analyzed by computer program SPSS" ver. 20" Chicago. USA. Data expressed as mean,
99 standard deviation and percentage. Whereas the cut-off point, sensitivity and specificity were made using
100 MedCalc program. Prior to analysis the variables were tested for normal distribution using the Shapiro-Wilk
101 Wtest. Student t-test was used for normally distributed data and Mann-Whitney was used for skewed data for
102 the purpose of identifying differences between the tested groups. ANOVA test was used for comparison
103 between different groups. Differences were considered significant at $p \leq 0.05$. Chi Square (χ^2) was used to
104 determine significance for categorical variables. Spearman correlation was used for correlations between
105 groups.

106 3. RESULTS

107 The demographic and clinical data of patients and controls of the current study were clarified in table (1). The
108 age of breast cancer disease group was significantly higher than benign breast diseases. The age at menarche
109 and age at menopause were not relevant to either benign breast diseases or breast cancer diseases groups.
110 The mean levels of BMI were significantly higher in benign and breast cancer groups in comparison to those of
111 controls. Family history of breast cancer and history of contraception in patients and controls were shown in
112 tables (1). Table (2) showed the clinicopathological characteristics of the breast cancer patients.

113 There were 26 (66.7%) of 39 BC patients had positive Caveolin 1 gene as shown in fig. (1). Figure (2) showed
114 positive and negative samples for the presence of caveolin1 gene by agarose gel electrophoresis. The
115 statistical relationship between clinicopathological features and other molecular markers with caveolin-1 gene
116 analysis in breast cancer patients were shown in table (3). Caveolin-1 gene was associated with larger tumor
117 size ($p < 0.05$), grade ($p < 0.05$), advanced stage ($p < 0.01$) and lymphovascular invasion ($p < 0.05$).

118 Table (4) showed the levels of studied parameters in patients and controls. The current study showed a high
119 significant difference ($p < 0.001$) in levels of hyaluronan, CA 15-3 between breast cancer patients before
120 operation and control groups and between breast cancer patients before operation and benign breast diseases
121 groups. The mean $\pm$ SD levels of serum hyaluronan in breast cancer patients showed a decrement towards
122 normalization of levels 6 month after the surgical removal of their tumors and after they received medical
123 treatment. After 12 months, hyaluronan levels showed no significant difference from pretherapy levels. Also,
124 CA 15-3 levels were changed towards normalization, 12 months after treatment.

125 The sensitivity and specificity based on the cut-off levels for studied variables in breast cancer patients were
126 demonstrated in tables (5).

127 The result of the current study showed significant relation between hyaluronan and clinicopathological
128 characteristic of breast cancer patients before operation as regarding axillary lymph node involvement and
129 distant metastasis (table 6). Interestingly, only CA 15-3's correlation with lymphovascular invasion was
130 statistically significant.

131 The relation between the Caveolin-1 gene analysis and biochemical parameters among breast cancer patients
132 were presented in Table (7). Caveolin1 gene showed significant associations with serum CA 15-3 levels in
133 breast cancer patients.

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Table 1. The demographic and the clinical data of patients and controls.

Variables	Controls (n=10)	Benign breast diseases group (n=10)	Breast cancer (group I) (n=39)
Age (years) mean $\pm$ SD	44.20 $\pm$ 10.81	36.30 $\pm$ 9.14 p1 NS	50.67 $\pm$ 10.32 p1 NS p2 < 0.01
Age at menarche (years) mean $\pm$ SD	13.10 $\pm$ 1.28	13.30 $\pm$ 1.25 p1 NS	13.46 $\pm$ 1.62 p1 NS p2 NS
Age at menopause (years) mean $\pm$ SD	52.25 $\pm$ 3.20	48.50 $\pm$ 2.12 p1 NS	48.74 $\pm$ 3.4 p1 NS p2 NS
Body mass index (kg /m2) mean $\pm$ SD	29.20 $\pm$ 2.66	32.97 $\pm$ 6.93 p1 < 0.05	32.84 $\pm$ 5.20 p1 < 0.05 p2 NS
Family history of breast cancer N (%)			
-Yes	1 (10.0%)	1 (10.0%)	4 (10.3%)
-No	9 (90.0%)	9 (90.0%)	35 (89.7%)
History of using contraceptives N (%)			
-Yes	6 (60.0%)	4 (40.0%)	21 (53.8%)
-No	4 (40.0%)	6 (60.0%)	18 (46.2%)

Group I: Breast cancer patients at diagnosis.

p1: Compared to controls, p2: Compared to benign breast diseases group, NS: non-significant.

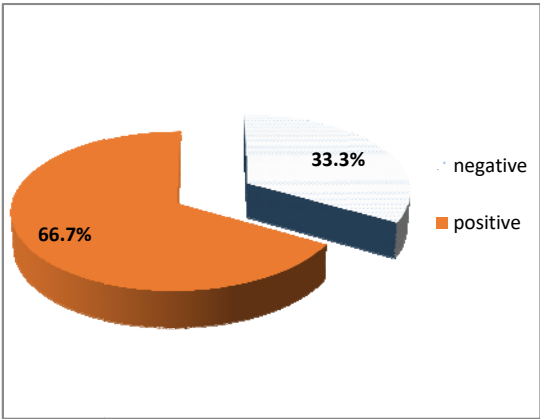
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Table 2. The clinicopathological characteristics of the breast cancer patients.

	Number of patients	Percentage
Grade:		
- Grade I	2	5.1%
- Grade II	24	61.5%
- Grade III	13	33.3%
Tumor size (T)		
- T1	5	12.8%
- T2	24	61.5%
- T3	9	23.1%
- T4	1	2.6%
Regional L.N.		
- N0	10	25.6%
- N1	15	38.5%
- N2	8	20.5%
- N3	6	15.4%
Distant metastasis (M)		
- M0	33	84.6%
- M1	6	15.4%
Stages		
- I	3	7.7%
- II	21	53.8%
- III	10	25.6%
- IV	5	12.8%
Estrogen Receptor (ER)		
-Positive	20	51.3%
-Negative	18	46.2%
-Not available	1	2.5%
Progesterone Receptor (PR)		
- Positive	14	35.9%
- Negative	24	61.5%
- Not available	1	2.6%
HER2\ neu		
- Positive	8	20.5%
- Negative	14	35.9%
- Not available	17	43.6%
Distribution of breast cancers		
- Right-sided tumor	18	46.2%
- Left-sided tumor	21	53.8%
Lymphovascular invasion		
- Yes	25	64.1%
- No	14	35.9%
Type of surgery		
- Modified radical mastectomy (MRM)	24	61.6%
- Conservative breast surgery (CBS)	10	25.6%
- Palliative mastectomy	5	12.8%
Menopausal state		
- Pre-menopausal	16	41%
- Post-menopausal	23	59%

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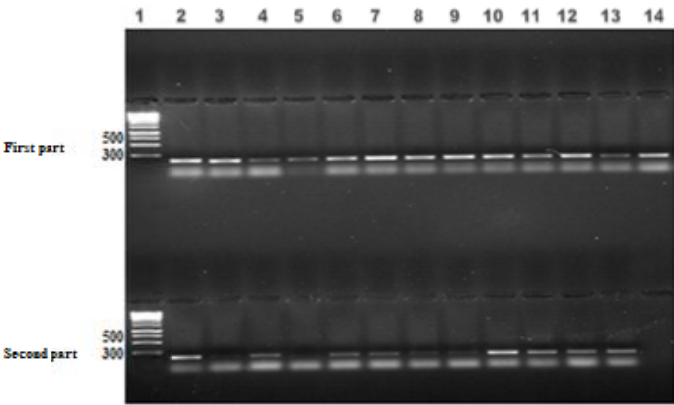
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Fig.1. Percentage of caveolin1 gene in breast cancer group before operation



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Fig. 2. Agarose gel electrophoresis (1.5%) for PCR products of caveolin 1 gene. Lane 1 represented DNA marker. The first part: Lanes 2-14 represented positive samples for the presence of caveolin1 gene (100 bp PCR products). The second part: Lanes 3, 5, 8, 9 represented negative samples for caveolin1 gene, other lanes were positive for the gene. The faint band indicated decrease gene expression which correlated with early stage

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Table 3. Statistical relationship between caveolin 1 gene analysis and the studied clinicopathological characteristics of the breast cancer patients.

Variables	Total N (39)	Caveolin 1 gene positive cases (n=26) {N (%)}	Caveolin1 gene negative cases (n=13) {N (%)}	p value
Grade:				
-Grade I	2	0 (0.0%)	2 (100%)	p<0.05
-Grade II	24	19 (79.2%)	5 (20.8%)	
-Grade III	13	7 (53.8%)	6 (46.2%)	
Tumor size				
-T1	5	1(20%)	4 (80%)	p<0.05
-T2	24	16 (66.7%)	8 (33.3%)	
-T3	9	8 (88.9%)	1(11.1%)	
-T4	1	1(100%)	0 (0.0%)	
Regional L.N.				
-N0	10	5 (50%)	5 (50%)	NS
-N1	15	8 (53.3%)	7 (46.7%)	
-N2	8	8 (100%)	0 (0.0%)	
-N3	6	5 (83.3%)	1 (16.7%)	
Distant metastasis (M)				
-M0	33	20 (60.6%)	13 (39.4%)	NS
-M1	6	6 (100%)	0 (0.0%)	
Stages				p<0.01
-I	3	0 (0.0%)	3 (100%)	
-II	21	12 (57.1%)	9(42.9%)	
-III	10	9 (90%)	1 (10%)	
-IV	5	5 (100%)	0 (0.0%)	
Estrogen Receptor				
-Positive	20	12(60%)	8 (40.9%)	NS
-Negative	18	14 (77.8%)	4 (22.2%)	
-Not available	1	0 (0.0%)	1 (100%)	
Progesterone Receptor				
-Positive	14	9 (64.3%)	5 (35.7%)	NS
-Negative	24	17 (70.8%)	7 (29.2%)	
-Not available	1	0 (0.0%)	1 (100%)	
HER2\ neu				
-Positive	8	3 (37.5%)	5 (62.5%)	NS
-Negative	14	12 (85.7%)	2 (14.3%)	
-Not available	17	11 (64.7%)	6 (35.3%)	
Triple negative				
-Yes	11	9 (81.8%)	2 (18.2%)	NS
-No	28	17 (60.7%)	11(39.3%)	
Age				
- ≤50 years	19	11 (57.9%)	8 (42.1%)	NS
- >50 years	20	15 (75%)	5 (25%)	
Pathological tumor type				
-Carcinoma insitu	1	0 (0.0%)	1 (100%)	NS
-Invasive ductal	37	26 (70.3%)	11 (29.7%)	
-Atypical medullary carcinoma	1	0 (0.0%)	1 (100%)	
Lymphovascular invasion				
- Yes	25	20 (80%)	5 (20%)	p<0.05
- No	14	6 (42.9%)	8 (57.1%)	
Body mass index (BMI)				
- <30	11	7 (63.6%)	4 (36.4%)	
- ≥30	28	19 (67.9%)	9 (32.1%)	

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Table 4. The levels of studied parameters in patients and controls.

	Controls	Benign breast diseases group	Group I	Group II	Group III	ANOVA p-value
Variables	(n=10)	(n=10)	(n=39)	(n=17)	(n=9)	
Serum hyaluronan (ng/l)						
range	125-146	113-165	106-368	110-205	117.8-239.9	< 0.001
mean ± SD	135.5±7.4	128.54±15.04	199.5±67.4	156.8±30.48	190±37.2	
		*a***c	***ab	*bc	**ab	
Serum cancer antigen15-3 (U/ml)						
range	14-40	16-42	22.34-100	10.57-62	14-50	< 0.001
mean ± SD	25.2± 7.5	24.20 ±8.91	57.1±24.6	33.05±15.5	30.60±11.2	
		***c	***ab	**c	**c	

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Group I, II, III correspond to breast cancer patients at diagnosis, 6 months after treatment, 12 months after treatment respectively.

a: Compared to controls, b: Compared to benign breast diseases group, c: Compared to breast cancer group at diagnosis, *p<0.05, **p<0.01, ***p<0.001

Table 5. Cut-off points, sensitivity, specificity and area under ROC curve of studied parameters in breast cancer patients.

Variables	Cut-off (discriminant analysis)	Sensitivity %	Specificity %	Area under ROC curve
Serum hyaluronan (ng/l)	144. 6	79	90	0.877
CA 15-3 (U/ml)	30.2	87.18	80	0.926

Table 6. Statistical relation between the studied biochemical parameters and clinicopathological characteristics of the breast cancer patients before operation.

Variables	Number of patients	hyaluronan	CA 15-3
Grade			
- G1 + G2	26	196.5±64.8	56±23.5
- G3	13	205.6±74.7	59.4±27.6
TNM stage			
-I + II	24	175.4±45 ^b	51.5±23.7
-III + VI	15	238.2±79.9	66 ±24.2
Tumor size			
-T1+T2	29	194.9±62	54.5±22.7
-T3+T4	10	213±82	64.6±29.7
Axillary lymph node involvement			
-Negative	10	163±33.5 ^a	50±24
-Positive	29	212±71.9	59±24
Pathological tumor type			
- Ductal invasive	37	200±68.9	57.7±24.9
- Other tumor type	2	189±36	47±22
Distant metastasis			
- Yes	6	276±66 ^b	68±31.4
- No	33	185.6±58	55±23.3
Age			
- ≤50 years	19	195.5±71	52±20.3
- >50 years	20	203.4±65	61.8±27.9
Lymphovascular invasion			
- Yes	25	214.5±77	64.9±23.9 ^b
- No	14	172.9±32	43.3±20.2
Menopausal status			
-Premenopausal	23	202±71	51.9±21
-Postmenopausal	16	197±66	61.2±26.9
Estrogen Receptor			
-Positive	20	201±78	56±24.5
-Negative	18	197±56	58±25.5
-Not available	1		
Progesterone Receptor			
-Positive	14	203±88	58±27
-Negative	24	196.6±55	57.6±23.7
-Not available	1		
HER2\ neu			
-Positive	8	189.8±50	51.7±22.8
-Negative	14	216.6±70	60.8±25
-Not available	17		56.6±26

a: $p < 0.001$

b: $p < 0.01$

c: $p < 0.05$

Table 7. Statistical relationship between the studied biochemical parameters and caveolin1 gene analysis of the breast cancer patients.

Variables	Caveolin1 gene positive cases (n=26) Mean $\pm$ SD	Caveolin1 gene negative cases (n=13) Mean $\pm$ SD	p value
Serum hyaluronan (ng/l)	210.6 $\pm$ 76.5	177.5 $\pm$ 37.8	NS
Serum CA 15-3 (U/ml)	62.53 $\pm$ 24.7	46.3 $\pm$ 21.8	p<0.05

4. DISCUSSION

Breast cancer detection relies commonly on mammography, which associated with diminished breast cancer mortality [12]. However, mammography screening has made controversy due to the hazards of false-positive outcomes and over finding of indolent disease [13]. There is thus a critical requisite to recognize the biochemical bases of carcinogenesis, invasion and metastasis. The adding of a blood-based tumor indicator test may raise patient agreement as blood testing is more suitable and would also avoid the difficulties related with imaging high-density breast tissue [1].

In spite of the discovery of a number of breast cancer susceptibility genes in the last decades (e.g. TP53, PTEN, CDH1, STK11, CHEK2, ATM, BRIP1, BRCA1 and BRCA2), there are many genetic variants and triggers required to be recognized [14]. The present study found caveolin-1 gene in 26 (66.7%) of 39 cases of breast cancer group. On the other hand, caveolin-1 gene could not be detected in 33.3% of malignant cases. This may be due to decreased Cav-1 gene expression in non-metastatic primary tumors [15]. No Caveolin-1 gene could be found in benign breast disease group (n=10) and control group (n=10). These results agreed with Thompson et al. [16] and Chung et al. [17].

Lamaze and Torrino[18] found a strong association among Cav-1 expression and a basal like phenotype of breast cancer, as 52% of cancers that expressed Cav-1 had this phenotype, linked with only 9% of Cav1 negative carcinomas. In addition, 90% of metaplastic breast cancers revealed Cav1 expression.

Caveolin-1 can prevent apoptosis through numerous signal pathways and develops breast cancer cell survival. It is associated with cell transformation, cancer growth, cell migration, invasion and angiogenesis. In addition, Cav-1 shields cancer cells from death by speeding aerobic glycolysis[19]. The gene can facilitate multidrug resistance (MDR) by positively regulating the action of the ATP-binding cassette transporter breast cancer resistance protein in breast cancer [20]. Caveolin-1 knockdown could significantly decrease the tumorigenicity of breast cancer stem cells by downregulating the β -catenin/ ATP-binding cassette subfamily G member 2 signaling pathway [21]. Moreover, breast cancer violence is associated with Cav-1 CGI shore methylation levels, a newly described areas that flank CpG islands with less CG-density[22].

A scaffolding amino acid sequence identified in Cav-1 permits this protein to interact with signaling molecules, such as epidermal growth factor receptor (EGFR), G-proteins, Src family tyrosine kinases, Rho-GTPases, protein kinase C, eNOS, and integrin. In several types of cancer cells, Cav-1 is linked or co-localized with EGFR and appears to modify EGFR signaling[23]. Cav-1 can adjust these signaling molecules, thus playing a dynamic role in cancer advancement. The cooperation between cav-1 and Rho-GTPases helps tumor metastasis, which mostly depend on the raised expression of α 5-integrin and the higher activation of Src, Ras and Erk. Besides, an amplified expression of Cav-1 can stimulate the activation of AKT1, leading to the increased phosphorylation of Rho- GTPase. As a consequence, the invasion capability of breast cancer cells is significantly raised [20]. Also, cav-1 contributes in the remodelling of the extracellular matrix by promoting communications with matrix metalloproteinases [24].

272 In contrast, Chiu et al. [25] revealed that Cav-1 overexpression can decrease the primary breast cancer growth
273 and brain metastasis via the oncoprotein signal transducer and activator of transcription 3 (Stat3)
274 inhibition. Furthermore, Thomas et al. [26] showed Cav-1 downregulation (compared with normal tissue) in
275 breast cancer cells and demonstrated that loss of Cav-1 expression was linked with tamoxifen resistance.

276 Six months after treatment, caveolin-1 gene was detected in 5 of 17 cases (2 of them from stage II, 3 were from
277 stage III) due to difference in response of patients to treatment. Twelve months after treatment, no caveolin-
278 1 gene was observed in the followed up breast cancer women (n=9). Caveolin-1 gene modifies breast cancer
279 response to chemotherapy and radiation therapy [19]. On the other hand, Chatterjee et al. [21] reported that
280 radiation and chemotherapy up-regulate Cav-1 expression, while Cav-1 reduction induces both
281 chemosensitization and radiosensitization through changed apoptotic and DNA repair signaling.

282 In the current study, caveolin-1 gene has been linked to increased tumor diameter ($p<0.05$), poor
283 histopathologic grade ($p<0.05$) in BC patients at diagnosis as were found in previous studies (Chatterjee et
284 al. [21] and Zhao et al. [27]). Also, Cav-1 level was observed to have association with advanced stage ($p<0.01$)
285 and lymphovascular invasion ($p<0.05$) as shown in Qian et al. [28] and Mao et al. [29] studies. These data
286 designate its involvement in BC as a possible molecular goal and its ability to expect the patient's response to
287 treatment. Cav-1 has been documented as a janus-faced tumor regulator in different types of cancers [29]. A
288 microarray analysis also reinforced Cav-1 as a metastasis-related gene by matching gene expression profiles
289 between poorly and highly invasive breast cancer cells [30].

290 Caveolin-1 gene found in ER negative (14 of 18) patients more than ER positive (12 of 20) patients. This in line
291 with Rao et al. [22] that reported that cav-1 level correlated with ER α -negative BC patients. Also, Liedtke et al.
292 [31] found Caveolin-1 positivity significantly linked with lack of ER, PR, and HER2 expression.

293 The tumor promotion by HA may be mediated in several ways. The first way is accumulation of HA creates
294 highly hydrated and gel-filled spaces in extra cellular matrices. The formation of the less dense matrix
295 separates collagen layers and enhances tumor cell migration and penetration through physical barriers in the
296 matrices [32]. The second way is the interaction of HA with its cell surface receptors CD44 and RHAMM
297 influenced cellular processes such as differentiated cell growth, migration, and invasion which support
298 carcinogenesis [7]. Elevated synthesis of HA is associated with enhanced cell proliferation and
299 angiogenesis, and subsequently tangled in carcinogenesis [33].

300 In the current study, the mean serum hyaluronan levels were significantly higher in breast cancer women as
301 compared to that of controls and benign groups (table 4). These results are in accordance with the finding of
302 Yahya et al. [34] and Wu et al. [35]. Hyaluronic acid may be formed by tumor cells or as a consequence of
303 interactions between cancer and the adjacent connective tissue.

304 The present study also showed that the mean serum hyaluronan levels were significantly decreased ($p<0.05$)
305 in BC women after 6 months of surgical removal of tumors besides medical treatment. These results are in
306 accordance with the finding of Yahya et al. [34] and Peng et al. [36] which established the blocking of HA roles
307 by FAC chemotherapy have therapeutic significance in breast malignancy.

308 However, there was non-significant difference between levels of serum hyaluronan of pretreated BC women
309 and patients 12 months after treatment. This may be due to non-evident distant metastasis and/or non-specific
310 rise of HA. High serum HA has been reported with liver disease and various inflammatory conditions, including
311 rheumatoid arthritis, psoriasis, scleroderma, and osteoarthritis. Although, this biomarker is not specific to BC
312 diagnosis, serum HA levels may be useful to recognize potentially high-risk sets for additional diagnostic work-
313 ups or nearer follow-ups [37].

314 There is a significant correlation between serum hyaluronan and TNM stage ($p<0.01$) as also found by Corte et
315 al. [38] and Wu et al. [35]. Serum hyaluronan was correlated with lymph node positivity ($p<0.05$) and distant
316 metastasis ($p<0.01$) as shown by Auvinen et al. [39]. These results suggest that HA achieves a number of roles
317 in developing tumors and especially contributes to invasion in initial and advanced stage breast cancer [40]. In
318 the contrary, Wu et al. [35] found no relationship between the serum HA levels and BC metastasis.

319 High preoperative CA 15-3 level is directly linked to tumor burden. CA15-3 is possibly the best identified, non-
320 invasive marker of breast cancer, even though its suggested medical use is limited to checking of patients with
321 metastatic disease during treatment [41]. This indicator plays the biological roles such as cell connection,
322 immunity and it is responsible for metastasis [42]. So it can be used in combination with investigative imaging
323 and history, physical examination in assessing return of disease and response to treatment [43].

Concerning CA 15-3, the present study found that its preoperative levels in breast cancer patients were significantly higher than control group and benign group (table 4) with no significant difference between control and benign groups. Similar observations were reported by previous studies (Gautam et al.[43]and Nieder et al. [9]). CA 15-3 levels decreased significantly after 6, 12 months of operation and treatment to be non-significant from control and benign group levels. These findings are in line with Gautam et al.[43]and Khan et al.[41]. The only clinicopathological feature related to elevated CA 15-3 levels was lymphovascular invasion. In addition, CA 15-3 levels was found to be increased across different stages (stage II 49 ± 17 U/ml, stage III 73.5 ± 26 U/ml, stage IV 99.2 U/ml) but did not reach a statistically significant level. Lee et al. [44]demonstrated that raised CA 15-3 before surgery was significantly associated with tumor size, axillary node involvement and advanced stage. On the other hand, Geng et al.[45]found a correlation with metastatic sites. The result was in agreement with previous studies (Incoronato et al.[46]and Śliwowska et al. [47]).

This study revealed significant correlation between cav-1 gene and serum CA 15-3 ($p<0.05$) levels in breast cancer patients. This association may indicate their associated role in breast cancer formation, invasion and metastasis.

5. CONCLUSIONS

The current study revealed that presence of caveolin-1gene, high circulating hyaluronan and CA 15-3 are significantly associated with breast carcinogenesis and act together as a battery for tumor progression and its metastasis. Accordingly, estimation of these biomarkers may expect the breast disease behavior and its prognosis and throw the light for the future use of these biomarkers as ideal chemopreventive or therapeutic agents for breast cancer.

COMPETING INTERESTS

Authors have reported that no competing interests exist.

REFERENCES

1. González-Díaz C, Uscanga-Carmona M, Lozano-Trenado L, Ortiz J, González J, Guerrero-Robles C, editors. Clinical Evaluation of Inductive Spectrometer to detect Breast Cancer. VII Latin American Congress on Biomedical Engineering CLAIB 2016, Bucaramanga, Santander, Colombia, October 26th-28th, 2016; 2017: Springer.
2. Naz N, Khanum S, Dal Sasso GTM, de Souza MdL. Women's Views on Handling and managing their breast cancer in Pakistan: A qualitative study. Diseases. 2016;4(2):17.
3. Zeeneldin AA, Ramadan M, Elmashad N, Fakhr I, Dīaa A, Mosaad E. Breast cancer laterality among Egyptian patients and its association with treatments and survival. Journal of the Egyptian National Cancer Institute. 2013;25(4):199-207.
4. Kazarian A, Blyuss O, Metodieva G, Gentry-Maharaj A, Ryan A, Kiseleva EM, et al. Testing breast cancer serum biomarkers for early detection and prognosis in pre-diagnosis samples. British Journal of Cancer. 2017;116(4):501-8.
5. Joglekar M, Elbazanti WO, Weitzman MD, Lehman HL, van Golen KL. Caveolin-1 Mediates Inflammatory Breast Cancer Cell Invasion via the Akt1 Pathway and RhoC GTPase. Journal of cellular biochemistry. 2015;116(6):923-33.
6. Lu Y, Li X, Liang K, Luwor R, Siddik ZH, Mills GB, et al. Epidermal growth factor receptor (EGFR) ubiquitination as a mechanism of acquired resistance escaping treatment by the anti-EGFR monoclonal antibody cetuximab. Cancer Research. 2007;67(17):8240-7.
7. Stivarou T, Patsavoudi E. Extracellular molecules involved in cancer cell invasion. Cancers. 2015;7(1):238-65.
8. Kabel AM, Al-shehri AH, Madani BS, Al-shafie SI, Amasha SA. Tumor Markers of Breast Cancer: Role in Early Diagnosis, Monitoring Response to Therapy and Determination of Prognosis. Journal of Cancer Research and Treatment. 2016;4(5):80-7.
9. Nieder C, Dalhaug A, Haukland E, Mannsaker B, Pawinski A. Prognostic Impact of the Tumor Marker CA 15-3 in Patients With Breast Cancer and Bone Metastases Treated With Palliative Radiotherapy. Journal of Clinical Medicine Research. 2017;9(3):183.

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373 10. Månsson B, Carey D, Alini M, Ionescu M, Rosenberg LC, Poole AR, et al. Cartilage and bone
 374 metabolism in rheumatoid arthritis. Differences between rapid and slow progression of disease identified by
 375 serum markers of cartilage metabolism. *Journal of clinical investigation*. 1995;95(3):1071.
 376 11. Duffy MJ, Evoy D, McDermott EW. CA 15-3: uses and limitation as a biomarker for breast cancer.
 377 *Clinica Chimica Acta*. 2010;411(23):1869-74.
 378 12. Gøtzsche PC, Jørgensen KJ. Screening for breast cancer with mammography. *The Cochrane Library*.
 379 2013.
 380 13. Welch HG, Prorok PC, O'Malley AJ, Kramer BS. Breast-cancer tumor size, overdiagnosis, and
 381 mammography screening effectiveness. *New England Journal of Medicine*. 2016;375(15):1438-47.
 382 14. Cardoso F, Harbeck N, Barrios C, Bergh J, Cortés J, El Saghir N, et al. Research needs in breast
 383 cancer. *Annals of Oncology*. 2016;mdw571.
 384 15. Sloan EK, Ciocca DR, Pouliot N, Natoli A, Restall C, Henderson MA, et al. Stromal cell expression of
 385 caveolin-1 predicts outcome in breast cancer. *The American journal of pathology*. 2009;174(6):2035-43.
 386 16. Thompson DE, Siwicki MD, Moorehead RA. Caveolin-1 expression is elevated in claudin-low
 387 mammary tumor cells. *Cancer cell international*. 2012;12(1):1.
 388 17. Chung Y-C, Kuo J-F, Wei W-C, Chang K-J, Chao W-T. Caveolin-1 dependent endocytosis enhances
 389 the chemosensitivity of HER-2 positive breast cancer cells to trastuzumab emtansine (T-DM1). *PloS one*.
 390 2015;10(7):e0133072.
 391 18. Lamaze C, Torrino S. Caveolae and cancer: A new mechanical perspective. *Biomedical journal*.
 392 2015;38(5):367.
 393 19. Pucci M, Bravata V, Forte GI, Cammarata FP, Messa C, Gilardi MC, et al. Caveolin-1, Breast Cancer
 394 and Ionizing Radiation. *Cancer Genomics-Proteomics*. 2015;12(3):143-52.
 395 20. Fu P, Chen F, Pan Q, Zhao X, Zhao C, Cho WC-S, et al. The different functions and clinical
 396 significances of caveolin-1 in human adenocarcinoma and squamous cell carcinoma. *OncoTargets and*
 397 *therapy*. 2017;10:819.
 398 21. Chatterjee M, Ben-Josef E, Thomas DG, Morgan MA, Zalupski MM, Khan G, et al. Caveolin-1 is
 399 associated with tumor progression and confers a multi-modality resistance phenotype in pancreatic cancer.
 400 *Scientific reports*. 2015;5.
 401 22. Rao X, Evans J, Chae H, Pilrose J, Kim S, Yan P, et al. CpG island shore methylation regulates
 402 caveolin-1 expression in breast cancer. *Oncogene*. 2013;32(38):4519-28.
 403 23. Sugie S, Mukai S, Yamasaki K, Kamibepu T, Tsukino H, Kamoto T. Significant Association of
 404 Caveolin-1 and Caveolin-2 with Prostate Cancer Progression. *Cancer Genomics-Proteomics*. 2015;12(6):391-
 405 6.
 406 24. Senetta R, Stella G, Pozzi E, Sturli N, Massi D, Cassoni P. Caveolin-1 as a promoter of tumour
 407 spreading: when, how, where and why. *Journal of cellular and molecular medicine*. 2013;17(3):325-36.
 408 25. Chiu W-T, Lee H-T, Huang F-J, Aldape KD, Yao J, Steeg PS, et al. Caveolin-1 upregulation mediates
 409 suppression of primary breast tumor growth and brain metastases by stat3 inhibition. *Cancer research*.
 410 2011;71(14):4932-43.
 411 26. Thomas NB, Hutcheson IR, Campbell L, Gee J, Taylor KM, Nicholson RI, et al. Growth of hormone-
 412 dependent MCF-7 breast cancer cells is promoted by constitutive caveolin-1 whose expression is lost in an
 413 EGF-R-mediated manner during development of tamoxifen resistance. *Breast cancer research and treatment*.
 414 2010;119(3):575-91.
 415 27. Zhao X, Yu G, Yu X, Wang J, Pan X. Caveolin-1 is overexpressed in hypopharyngeal squamous cell
 416 carcinoma and correlates with clinical parameters. *Oncology Letters*. 2016;12(4):2371-4.
 417 28. Qian N, Ueno T, Kawaguchi-Sakita N, Kawashima M, Yoshida N, Mikami Y, et al. Prognostic
 418 significance of tumor/stromal caveolin-1 expression in breast cancer patients. *Cancer science*.
 419 2011;102(8):1590-6.
 420 29. Mao X, Wong SYS, Tse EYT, Ko FCF, Tey SK, Yeung YS, et al. Mechanisms through which hypoxia-
 421 induced caveolin-1 drives tumorigenesis and metastasis in hepatocellular carcinoma. *Cancer Research*.
 422 2016;76(24):7242-53.
 423 30. Wang Z, Wang N, Liu P, Peng F, Tang H, Chen Q, et al. Caveolin-1, a stress-related oncotarget, in
 424 drug resistance. *Oncotarget*. 2015;6(35):37135.
 425 31. Liedtke C, Kersting C, Bürger H, Kiesel L, Wülfing P. Caveolin-1 expression in benign and malignant
 426 lesions of the breast. *World journal of surgical oncology*. 2007;5(1):110.
 427 32. Li Y, Li L, Brown TJ, Heldin P. Silencing of hyaluronan synthase 2 suppresses the malignant
 428 phenotype of invasive breast cancer cells. *International journal of cancer*. 2007;120(12):2557-67.
 429 33. Tammi RH, Kultti A, Kosma V-M, Pirinen R, Auvinen P, Tammi MI, editors. *Hyaluronan in human*
 430 *tumors: pathobiological and prognostic messages from cell-associated and stromal hyaluronan*. *Seminars in*
 431 *cancer biology*; 2008: Elsevier.

34. Yahya RS, El-Bindary AA, El-Mezayen HA, Abdelmasseh HM, Eissa MA. Biochemical evaluation of hyaluronic acid in breast cancer. *Clin Lab*. 2014;60(7):1115-21.10.
35. Wu M, Cao M, He Y, Liu Y, Yang C, Du Y, et al. A novel role of low molecular weight hyaluronan in breast cancer metastasis. *The FASEB Journal*. 2015;29(4):1290-8.
36. Peng C, Wallwiener M, Rudolph A, Čuk K, Eilber U, Celik M, et al. Plasma hyaluronic acid level as a prognostic and monitoring marker of metastatic breast cancer. *International journal of cancer*. 2016;138(10):2499-509.
37. Aghcheli K, Parsian H, Qujeq D, Talebi M, Mosapour A, Khalilipour E, et al. Serum hyaluronic acid and laminin as potential tumor markers for upper gastrointestinal cancers. *European journal of internal medicine*. 2012;23(1):58-64.
38. Corte MD, González LO, Junquera S, Bongera M, Allende MT, Vizoso FJ. Analysis of the expression of hyaluronan in intraductal and invasive carcinomas of the breast. *Journal of cancer research and clinical oncology*. 2010;136(5):745-50.
39. Auvinen P, Tammi R, Kosma VM, Sironen R, Soini Y, Mannermaa A, et al. Increased hyaluronan content and stromal cell CD44 associate with HER2 positivity and poor prognosis in human breast cancer. *International journal of cancer*. 2013;132(3):531-9.
40. Schwertfeger KL, Cowman MK, Telmer PG, Turley EA, McCarthy JB. Hyaluronan, inflammation, and breast cancer progression. *Frontiers in immunology*. 2015;6.
41. Khan MA, Trivedi HP, Atara AN. Analysis of tumour marker CA 15-3 in breast cancer following surgery. *International Surgery Journal*. 2016;3(3):1491-4.
42. Moazzezy N, Farahany TZ, Oloomi M, Bouzari S. Relationship between preoperative serum CA 15-3 and CEA levels and clinicopathological parameters in breast cancer. *Asian Pac J Cancer Prev*. 2014;15(4):1685-88.
43. Gautam A, Verma S, Pantola C, Verma S. Utility of CA 15-3 as Diagnostic and Prognostic Marker in BreastCancer. *IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)*. 2015;1(14):17-20.
44. Lee J, Park S, Park J, Cho J, Kim S, Park B-W. Elevated levels of preoperative CA 15-3 and CEA serum levels have independently poor prognostic significance in breast cancer. *Annals of oncology*. 2012;mds604.
45. Geng B, Liang M-M, Ye X-B, Zhao W-Y. Association of CA 15-3 and CEA with clinicopathological parameters in patients with metastatic breast cancer. *Molecular and clinical oncology*. 2015;3(1):232-6.
46. Incoronato M, Mirabelli P, Catalano O, Aiello M, Parente C, Soricelli A, et al. CA15-3 is a useful serum tumor marker for diagnostic integration of hybrid positron emission tomography with integrated computed tomography during follow-up of breast cancer patients. *BMC cancer*. 2014;14(1):356.
47. Śliwowska I, Kopczyński Z, Grodecka-Gazdecka S. Diagnostic value of measuring serum CA 15-3, TPA, and TPS in women with breast cancer. *Journal cover*. 2016;70.

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