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ORIGINAL ARTICLE

Assessment of Stefin-A Gene Expression in Breast Cancer Women

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ABSTRACT

Background: Breast cancer (BC) is a very malignant tumor that is responsible for most cancer-related fatalities in women all over the world. The objective of the present study was to evaluate the potential of circulating Stefin-A and cancer antigen 15-3 (CA15-3) as tumor biomarkers in localized BC patients and those with metastatic BC.

Method: The study was a case-control study, that included 108 Egyptian females who were classified into three groups: healthy control, localized BC, and metastatic BC groups. The Stefin-A relative expression level was measured in the blood utilizing quantitative real-time polymerase chain reaction (qRT-PCR) while the serum level of CA15-3 was measured by ELISA.

Results: Stefin-A expression was statistically significantly higher among BC patients than metastatic BC patients than the healthy group (p-value:0.001). Among localized BC patients, there was a statistically positive correlation between Stefin-A and CA15-3 ($r=0.58$ and $p<0.001$). While, among BC patients with metastasis, there was a statistically significant negative correlation between them ($r=-0.36$ and $p=0.03$).

Conclusion: According to our results, we indicate that Stefin-A is negatively associated with the progression, invasiveness, and lymphatic metastasis of BC, and strongly, Stefin-A may be useful in molecular diagnosis and gene therapy for BC.

Keywords: Breast Cancer, Metastasis, Stefin-A, CA15-3, Gene Expression.

INTRODUCTION

Breast cancer (BC) is a type of tumor that originates from glandular milk duct epithelial cells or breast lobules. It is the most prevalent cancer in women, accounting for 2.26 million new cases and 685,000 fatalities in 2020 [1].

The survival rate for BC is very low, but it can be improved with early detection and treatment [2]. Therefore, finding the genetic mutations that cause BC can help with diagnosis, prognosis, and treatment [3].

The cystatin superfamily is a group of proteins that share some structural features. It includes five families of strongly linked proteins: family I cystatins (stefins), family II cystatins, family III cystatins, kininogens, and some non-inhibitory proteins [4].

Cystatins are proteins that control the activity of cysteine peptidases, such as cathepsins, which can be involved in cancer. However, the equilibrium between cystatins and cysteine peptidases can be altered in many pathological events such as cancer formation and progression [5].

Stefins are non-glycosylated proteins and are found inside cells and in body fluids. Stefin-A is a single-chain protein having a molecular weight of 11 kDa and 98 amino acid residues [6].

Stefin-A was the first protein that inhibited cathepsins related to cancer. It was highly produced in both lymphoid tissues and epithelial cells, and it is intimately linked to the onset and progression of cancer [7].

The low levels of Stefin-A in breast, brain, lung, prostate, and esophagus cancers are linked to cancer development and progression [8]. Serum tumor markers are substances in the blood that can help diagnose cancer early, assess how well the treatment is working, predict how the cancer will respond to certain therapies, and detect recurrence following surgery [9].

Tumor markers are often used in cancer screening and surveillance. The FDA has approved two

tumor markers, CA15-3, and CEA, for monitoring BC [10]. CA15-3 is a protein fragment that is released into the blood and can be detected by many antibodies. It is the most common blood marker used to detect recurrent BC and to check how well the treatment is working for patients with advanced cancer [11]. CA15-3 can also be found in ovarian, breast, pancreatic, lung, and colon cancers [12].

In the current research, we analyzed Stefin-A and CA15-3 expression in the blood of BC patients and investigated the relationship between their expression and characteristics related to clinicopathology.

SUBJECTS AND METHODS

This research was a case-control study that involved 108 individuals classified into three groups: 36 healthy women without BC, 36 female patients with localized BC, and 36 female patients with metastatic BC. Using Epi-Tools Epidemiological calculators, the sample size was determined to assess the statistical strength of the results, which demonstrated a strength of at least 87.1% [13]. The study was conducted at Zagazig University Hospitals in Egypt between November 2022 and October 2023 within the Medical Biochemistry & Molecular Biology and General Surgery departments. The patients were non-obese Egyptian BC females who were histopathological and clinically diagnosed with an age range of 35-56 years. The Patients did not get radiation therapy or chemotherapy before surgery. Data regarding the patients' clinicopathology were obtained from the pathology and hospital reports. Patients with infectious or inflammatory or autoimmune disorders or multiple primary cancers, patients with a family history of BC, and patients who refused to give consent weren't allowed to participate in the study. The study protocol was approved by the Zagazig University Institutional Research Board (IRB) (ZU-IRB#10101/13-11-2022). The Helsinki Declaration, which is the World Medical Association's code of ethics for human research, was followed in conducting this study.

RNA Extraction and Reverse Transcription

Thermo Fisher Scientific, Inc.'s Trizol reagent was used to extract total RNA from blood samples. Using 1.5 µl of RNA and a NanoDrop® ND–1000 Spectrophotometer (NanoDrop Technologies; Wilmington, Delaware, United States), the A260/A280 ratio was measured to determine the quality of the RNA. For cDNA synthesis, a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, USA) was used in accordance with the manufacturer's instructions.

Quantitative Real-Time PCR

The real-time RT-PCR was carried out using TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Cat. # P725 or P750) (Enzynomics, Korea) in a Mx3005P Real-Time PCR System (Agilent Stratagene, USA) according to the manufacturer's instructions. An initial denaturation step at 95°C for 12 minutes was followed by 40 cycles of denaturation for 20 seconds at 95°C, annealing for 30 seconds at 60°C, and extension for 30 seconds at 72°C during the PCR cycling conditions. Sangon Biotech (Beijing, China) produced primers unique to oligonucleotides. Following PCR amplification, a melting curve analysis was carried out. The mRNA expression of a known housekeeping gene, human GAPDH, was used to normalize the expression level of the target gene. The outcomes are shown as fold-alterations made using the $2^{-\Delta\Delta CT}$ method in comparison to the control group [14]. Primer sequences were as follows: Stefin-A (173 bp) (forward); 5′-ATT ACT ACA TTA AGG TAC GAG CAGG-3′, (reverse); 5′-AAG GAA TCA GAA CAC TTT GGGT-3′, GAPDH (161 bp) (forward) 5′-GGA GTC AAC GGA TTT GGT CGT-3′, (reverse); 5′-ACG GTG CCA TGG AAT TTGC-3′.

ELISA Assay

The levels of human CA15-3 in serum were determined using the Human CA15-3 ELISA kit (INNOVA BIOTECH CO., LTD), which is a new and optimized ELISA kit The analysis was standardized according to the manufacturer's instructions, and the results were adjusted to a standard curve.

Statistical analysis

Data were gathered and assessed with IBM's Version 20.0 of SPSS software. Standard deviation, mean, range, and median were utilized to characterize quantitative data (IQR). Additionally, the Chi-square test, a one-way ANOVA test, and Turkey's test for multiple comparisons for three groups were used to analyze categorical data. Every test had two sides. According to a standard curve, a p-value of less than 0.05 was regarded as statistically significant, while a p-value greater than 0.05 was regarded as statistically insignificant.

RESULTS

The studied subjects' females were classified into three groups (control, localized BC patients, and BC patients with metastasis) with a mean age of; 44.3±8.3, 48.5±6.2, and 45.5±7.6, respectively. The group subjects were 55.6%, 44.4%, and 52.8% in premenopausal status, while 44.4%, 55.6%, and 47.2% were in a postmenopausal state, respectively. Regarding estrogen receptor (ER), progesterone receptor (PR), and human epidermal

growth factor-2 receptor (HER-2) status; 30.6%, 41.7%, 66.7% were negative in group II, and 69.4%, 58.3%, 33.3% were positive, while in group III 22.2%, 30.6%, 75% were negative, and 77.8%, 69.4%, 25% were positive. In group II 91.7% of cases were diagnosed as invasive ductal carcinoma and 8.3% were invasive lobular carcinoma. While in group III 88.9% were diagnosed with invasive ductal carcinoma and 11.1% were invasive lobular carcinoma. Regarding the tumor site, 41.7% of tumors were left-sided and 58.3% were right-sided in group II. While in group III 44.4% were left-sided, 52.8% were right-sided, and 2.8% were bilateral. In group II 77.8% were grades 1&2 and 22.2% were grade 3, while in group III 66.7% were grades 1&2 and 33.3.2% were grade 3. Regarding the tumor stage, 50% were T1&T2 and 50% were T3&T4 in group II, while in group III 61.1% were T1&T2 and 38.9% were T3&T4. 36.1% of group III cases had lymphatic metastasis only while 63.9% had lymphatic with distant metastasis.

Stefin-A expression was statistically significantly higher among BC patients than BC patients with metastasis than the control group (8.9±1.4>5.4±0.7>0.9±0.1, p-value: 0.001**) (Table 1).

There was a statistically significant difference between different stages of the tumor regarding Stefin-A gene expression in both groups (localized BC and metastatic BC) (p:0.02). Also, Stefin-A gene expressions were found to be significantly higher among patients with lymphatic metastasis only compared to patients with lymphatic and distant metastasis (p:0.01) (Table 2).

CA15-3 expression was statistically significantly higher among the metastasis BC group than the localized BC group than the healthy group (30.2±6.8>29.4±5.1>15.4±2.8, p-value:0.001**) (Table 1).

Among localized BC patients, there was a statistically positive correlation between Stefin-A and CA15-3 (r=0.58 and p<0.001) (Figure 1) and (Table 3). While, among patients with metastasis, there was a statistically significant negative correlation between Stefin-A and CA15-3 (r=-0.36 and p=0.03) (Table 3).

Stefin-A at a cutoff point of more than or equal to 2.2 can be used as a significant predictor for the presence of BC with a sensitivity of 100%, and specificity of 100% (Table 4) and (Figure 2). Also, Stefin-A at a cutoff point of less than or equal to 6.8 can be used as a significant predictor for the presence of metastasis in BC patients with a sensitivity of 100%, and specificity of 95% (Table 4) and (Figure 3).

CA15-3 at a cutoff point of more than or equal to 18.7 can be used as a significant predictor for the presence of BC with a sensitivity of 98.6%, and specificity of 83.3% (Table 5).

Table (1): Stefin-A relative mRNA expression and CA15-3 serum levels among the studied groups

Variable	Group I (Control) (n=36)	Group II (Localized BC) (n=36)	Group III (Metastatic BC) (n=36)	P*	LSD
Stefin-A: Mean ± SD Range	 0.9 ± 0.1 0.7 – 1.2	 8.9 ± 1.4 6.1 – 12.1	 5.4 ± 0.7 3.1 – 6.7	 **<0.001 (HS)**	**<0.05**[1] **<0.05**[2] **<0.05**[3]
CA15-3 (U/mL): Mean ± SD Range	 15.4 ± 2.8 10 – 19.8	 29.4 ± 5.1 19 – 40.2	 30.2 ± 6.8 18.5 – 43.3	 **<0.001 (HS)**	**<0.05**[1] **<0.05**[2] >0.05[3] (NS)

BC; breast cancer, CA15-3; cancer antigen 15-3, U/mL; unite per milliliter, *test; ANOVA test, LSD; Least significant difference post-hoc test, NS; non-significant difference (p>0.05), HS; highly significant difference (p<0.001). 1; Group I vs. Group II, 2; Group I vs. Group III, 3; Group II vs. Group III.

Table (2): Correlation between the clinicopathological data and Stefin-A relative expression in BC patients

Variable	N	Stefin-A	P
Menopause: Premenopausal Postmenopausal	 55 53	 4.9 ± 3.5 5.3 ± 3.3	 0.361 (NS)
Diagnosis: Invasive ductal carcinoma Invasive lobular carcinoma	 65 7	 7.2 ± 2 7.5 ± 2.6	 0.657 (NS)

Variable	N	Stefin-A	P
Site of tumor:			
Left	31	7 ± 2.1	0.695
Right	40	7.4 ± 2.1	(NS)
Bilateral	1	6.7 ± 0	
Grade of tumor:			
Grade I	8	7.1 ± 2.1	0.074
Grade II	44	7.6 ± 2.1	(NS)
Grade III	20	6.3 ± 1.7	
Stage of tumor:			
T1+T2	40	7.8 ± 2.3	**0.02**
T3+T4	32	6.7 ± 1.7	**(S)**
Metastasis:			
LN metastasis	13	5.8 ± 0.3	**0.01**
LN with distant metastasis	23	5.4 ± 0.5	**(S)**
ER:			
Negative	19	7.3 ± 2	0.717
Positive	53	7.1 ± 2.1	(NS)
PR:			
Negative	26	7.2 ± 1.8	0.896
Positive	46	7.2 ± 2.2	(NS)
HER-2:			
Negative	51	7.2 ± 2.1	0.947
Positive	21	7.2 ± 2.1	(NS)

BC; breast cancer, LN; lymph node, ER; estrogen receptor, PR; progesterone receptor, HER-2; human epidermal growth factor-2. NS; non-significant difference ($p>0.05$), S; significant difference ($p<0.05$).

Table (3): Correlation between Stefin-A expression and CA15-3 among the studied groups

Variable	Group II (Localized BC) (n=36)		Group III (Metastatic BC) (n=36)	
	Stefin-A		Stefin-A	
	r	p	r	p
CA15-3	0.58	<0.001	-0.36	0.03

Table (4): Performance of Stefin-A in predicting breast cancer and metastasis among the studied groups

Stefin-A	Cutoff-point	AUC	Sensitivity	Specificity	PVP	PVN	Accuracy
Predicting Breast Cancer	≥ 2.2	1.00	100%	100%	100%	100%	100%
Predicting Metastasis	≤ 6.8	0.991	100%	95%	94.7%	100%	97.2%

Table (5): Performance of CA15-3 in predicting breast cancer among the studied groups

Variable	Cutoff-point	AUC	Sensitivity	Specificity	PVP	PVN	Accuracy
CA15-3	≥ 18.7	0.993	98.6%	83.3%	92.2%	96.7%	93.5%

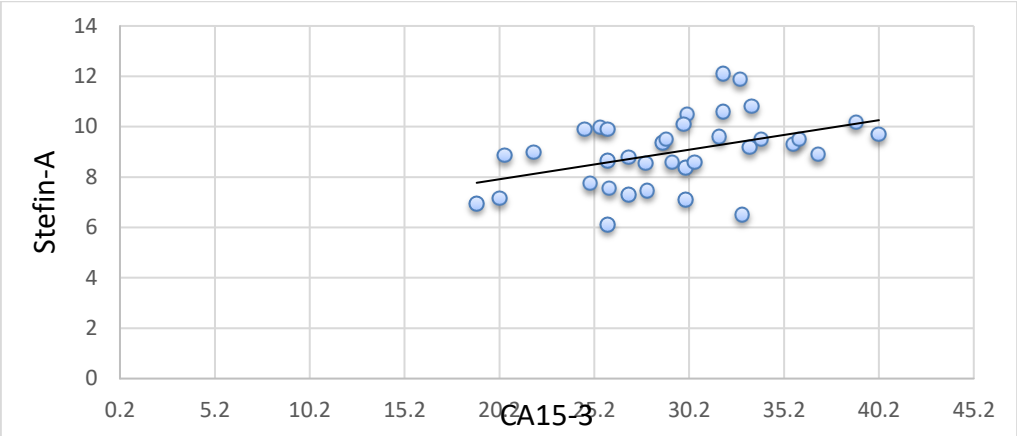

Figure 1: Correlation between Stefin-A and CA15-3 among breast cancer studied group.

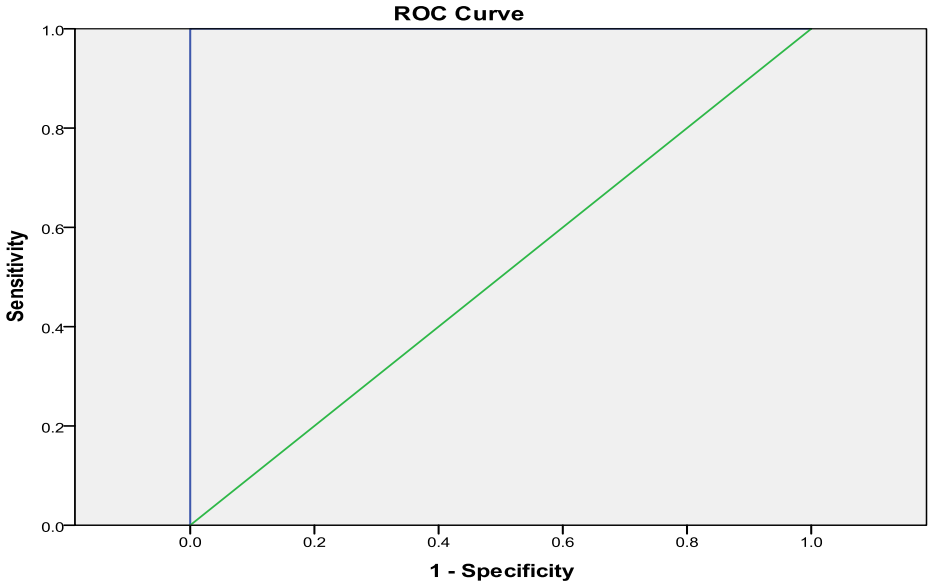

Figure 2: ROC curve for using Stefin-A as a predictor for breast cancer.

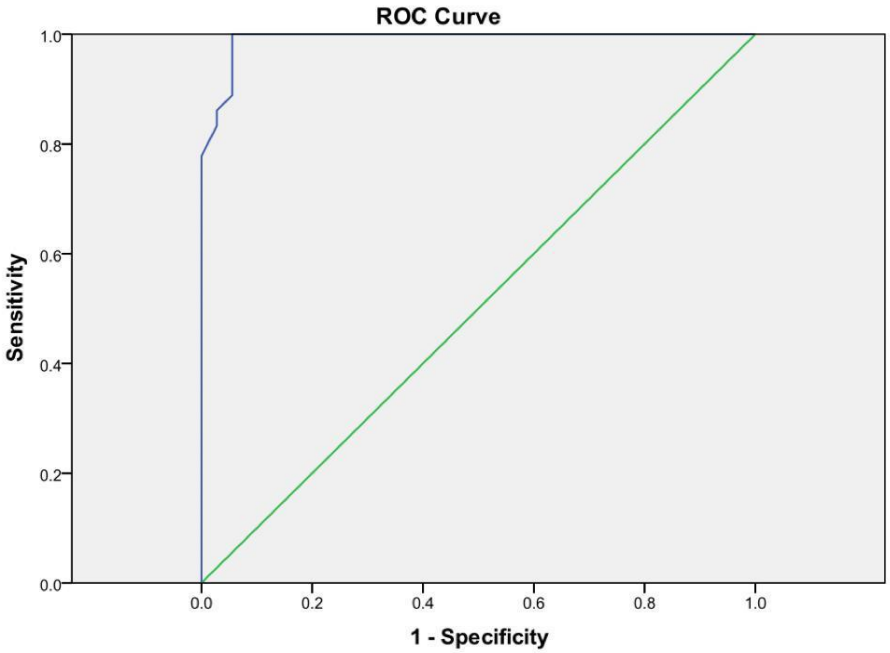

Figure 3: ROC curve for using Stefin-A as a predictor for breast cancer metastasis.

DISCUSSION

Biomarkers are essential in cancer research as they help doctors detect and measure the disease, estimate the chances of survival, and determine treatment response. They are usually obtained from less invasive samples such as blood, saliva, or urine [15].

The present study found that Stefin-A mRNA expression was statistically significantly higher in patients with localized BC (8.9±1.4-fold-change) than metastatic BC (5.4±0.7-fold-change) than normal individuals (0.9±0.1-fold-change) ($p<0.001$) (Table 1). The expression was highest significant among lower tumor stages (T1&T2) (p:0.02). Also, Stefin-A gene expression was found to be significantly higher among patients with lymphatic metastasis only compared to patients with lymphatic and distant metastasis (p:0.01) (Table 2). Serum CA15-3 levels were significantly higher among BC groups than the control group ($p<0.001$), but there was no significant difference between patients with localized or metastatic BC ($p>0.05$) (Table 1). Our results showed that among localized BC patients, there was a statistically positive correlation between Stefin-A and CA15-3 ($r=0.58$, $p<0.001$) (Figure 1) and (Table 3), while among patients with metastasis, there was a statistically significant negative correlation between Stefin-A and CA15-3 ($r=-0.36$, $p=0.03$) (Table 3).

When we plotted the ROC curve to investigate the ability of Stefin-A in the prediction of BC and metastasis, the test showed that monitoring Stefin-A expression in the blood could be a non-invasive and easy diagnostic procedure with 100% sensitivity and 100% & 95% specificity at a cut-off value of ≥2.2- & ≤6.8-fold change, with an area under the curve of 1.00 & 0.991 respectively (Table 4) and (Figure 2,3).

Guidelines from the American Society of Clinical Oncology state that 10% of patients with stage I, 20% of patients with stage II, 40% of patients with stage III, and 75% of patients with stage IV BC have high levels of CA15-3. Additionally, CA15-3 levels that are 5 to 10 times higher than the normal range may indicate the existence of metastatic illness [16]. Despite this, a low level of CA15-3 does not necessarily indicate the absence of metastasis. In fact, a small percentage of healthy people and patients with some benign conditions, most notably liver disease, can also have high CA15-3. Additionally, patients with different kinds of advanced adenocarcinomas may also have higher CA15-3 levels [17].

According to Molina et al, CA15-3 is not suitable for early detection or screening of breast cancer because it is not specific or sensitive enough. CA15-3 was only 16-18% sensitive in individuals suffering from local or regional disease and 61-70% sensitive among individuals with severe illness [18].

Stefins are proteins secreted from tumor cells and cells around tumors that can be detected in the fluids near tumors and in the urine and blood of cancer patients [19]. The variations in these proteins' levels outside the cells in the blood or secretions may be related to alterations in their production in cancer cells and the body's reaction to cancer and its spread [20].

Our results of Stefin-A are consistent with in vitro studies such as a study that used a mouse model of BC and a 3D culture system to investigate Stefin-A's role in preventing tumor cell invasion. The study found that Stefin-A was expressed a lot by myoepithelial cells, which are special cells that protect the milk ducts and lobules in the breast. They also showed that Stefin-A levels were lower in high-grade ductal carcinoma in situ (DCIS), which is a type of breast cancer that has not yet invaded, than in low-grade DCIS or normal breast tissue. This suggests that losing Stefin-A could be linked to a higher chance of invasive recurrence [21]. In consistency with our results, Parker et al. found that Stefin-A levels were lower in primary tumors that could spread more easily than in those that could not. They also found that Stefin-A levels were greater in microscopic metastasis but undetectable in macroscopic metastasis in the breast cancer model [22].

Lah et al. demonstrated that most breast cancers had lower levels of Stefin-A mRNA and protein, which could mean that Stefin-A plays a part in breast tumor development [23]. The growth of tumor cells with more aggressive and metastatic behavior may be due to a change in the stein-inhibiting action [24].

Stefin-A levels were greater in laryngeal cancer than in the normal tissue. Stefin-A levels were lower in patients with lymph node metastasis compared to those without. In contrast to our results, Stefin-A expression did not depend on the location, or differentiation of the tumors [25].

Also, Stefin-A levels in the blood were higher in hepatocellular carcinoma patients (HCC) and liver cirrhosis (LC) than in healthy people, and they were related to the size and number of tumors in patients. However, LC patients had much higher Stefin-A levels than HCC patients. In cancer patients, Stefin-A levels in the blood were shown to be significantly related to size and number of tumors [26].

Another study found that the Stefin-A mRNA expression in the HCC tissues was greater than in normal liver tissues or tissues around the cancer.

They also found that Stefin-A levels were related to the spread of cancer to the lymph nodes, and the size of the tumor [27]. Esophageal cancer cells overexpress Stefin-A, and Stefin-A cDNA transfection into these cells decreases tumor development, angiogenesis, invasion, and metastasis mostly via reducing cathepsin B activity [28].

Stefin-A can act as both a suppressor and an oncoprotein in different human cancers, and its reduced expression has been associated with a better prognosis in breast, liver, and brain malignancies [29]. In contrast with our findings, Kuopio et al. found that a high Stefin-A expression level was associated with metastatic BC, higher tumor grade, and a poor prognosis in patients and that the risk of BC-related death was significantly higher in patients with Stefin-A-positive tumors than in those with Stefin-A-negative tumors [30].

Additionally, Rudzinska-Radecka et al. found that Stefin-A expression was higher in renal cell carcinoma tissue samples compared to non-tumoral and benign tissue samples also found a positive correlation between Stefin-A expression and tumor size, grade, invasion of lymph nodes, and metastasis, which may suggest its role for the onset and progression of renal cell carcinoma [29].

We hypothesized that the highly complicated pathological processes underlying these diseases and the altered histology caused by malignancies account for the difference between our results and those of other researchers.

We acknowledge that the results reported here are preliminary, even if some of our data were statistically significant. This is because, in the first place, the sample size was rather small, and there was insufficient statistical power to investigate the true link. Second, no patients from outside of Zagazig University Hospitals were included in the patient pool. Third, ethnic variations could have a significant impact on this kind of genetic research. Fourth, there may be interactions between genes and the environment, making BC a complicated disease. Therefore, a single gene expression is not enough to fully explain the genetic component of BC.

Further genetic research of the Stefin-A gene expression with a bigger sample size is encouraged to learn more about its function in BC development and to assess the significance between them.

CONCLUSION

We found that in BC Egyptian female patients, there was a significant relationship between Stefin-A and CA15-3. The relative Stefin-A gene expression was greater in localized BC patients than metastatic BC patients and both were higher than the control group. The higher Stefin-A expression was also related to the lower tumor grades, stages, and BC with metastatic lymph nodes only rather than distant metastatic tumors. Therefore, it can serve as new biomarkers for the detection and evaluation of BC, assisting in the identification of BC patients in need of prompt treatment to lower their death rate and increase life expectancy. They can also be used as a target of therapy.

Ethical approval (including reference number)

The Ethical Board of the University of Zagazig, Faculty of Medicine, accepted this research (Zu-IRB# 10101/13-11-2022).

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