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Association of *SERPIND1* expression with grade, stage and presence of metastasis in breast cancer

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The role of *SERPIND1* gene in the development of breast cancer is completely unknown. The aim of the present study was to assess the expression of *SERPIND1* in patients suffering from breast malignancies. Tumoural specimens and non-tumoural adjacent tissues were collected from 31 patients who were pathologically confirmed for breast cancer. Furthermore, 43 fasting venous blood samples were collected from the breast cancer patients as well as from the healthy volunteers as control group. The *SERPIND1* mRNA expression levels were assessed using the q-RT PCR while the plasma levels of *SERPIND1* protein were detected using ELISA. The *SERPIND1* relative expression levels were compared with the clinicopathological factors. The expression of the *SERPIND1* was significantly higher at both mRNA and protein levels in breast cancer patients compared to the control groups. Moreover, the relative expression of the *SERPIND1* showed a significant positive correlation with cancer grade, stage and presence of metastasis ($P< 0.05$). The findings suggest that the *SERPIND1* may be a metastasis-enhancer factor in breast cancer and a strong predictive marker for distinguishing patients with poor prognosis.

Keywords: Breast Cancer, HCII, Heparin cofactor II, *SERPIND1*, Tumour marker

Breast cancer is the most common malignancy found in women. The disease is highly prevalent throughout the world[1-3]. Being the second most common cancer (after skin cancer), breast cancer encompasses 26% of all cancer cases in females. It is also the fifth most common cause of death due to cancer[2-5]. Although preventive measures or early detection programmes can reduce the mortality risk of breast cancer, no success is achieved in most of the cases. This is especially more pronounced in developing countries where the disease is mostly diagnosed in late stages[6]. Nowadays, mammography imaging modality is used as a gold standard method for diagnosis and screening of breast cancer. However, this method has limitations due to the use of ionizing radiation and low detection sensitivity (false positive rate of 8%-10%) and the fact that it is painful for the patients[7-9]. Cancer antigen (CA) 15-3 and carcinoembryonic antigen (CEA) are currently the most common tumour markers which are routinely used. Notably, CA15-3 has the highest sensitivity and specificity among these tumour markers[8-10]. However, the sensitivity of these markers in the detection of early-stage breast cancer is low; thus, more extensive studies are needed to find new, non-invasive markers with higher sensitivity and specificity[7,8].

SERPIND1, also known as heparin cofactor II (HCII), belongs to the serpin gene superfamily. It is a plasma glycoprotein and protease inhibitor which acts as a thrombin inhibitor while interacting with heparin, dermatan sulfate, and other endogenous glycosaminoglycans[10,11]. It is cytogenetically located on chromosome 22 (22q11. 21). *SERPIND1* contains 5 exons with a genomic size of 13608 bp. The highest expression of this gene has been detected in the liver tissue[12]. Over the past 30 years, *SERPIND1* has been periodically studied as a protease inhibitor. Recent assessments showed that the *SERPIND1* protein presents in the serum of patients with B-Cell acute lymphoblastic leukemia (B-ALL) can be used as a selected biomarker for early detection of B-ALL[13]. *SERPIND1* was overexpressed in the tumoural tissue compared to the normal tissue in the patients suffering

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from ovarian epithelial carcinoma[14]. The reports suggest that higher expression of *SERPIND1* in the tissues of ovarian carcinoma is associated with poor prognosis in the patients. Moreover, it seems that *SERPIND1* increases the ability of invasion and metastasis of lung cancerous cells[16]. Also, it was reported that *SERPIND1* plays a role in the metastasis of breast cancer to the brain[17]. In general, it seems that the expression of *SERPIND1* has a Stimulating effect on tumour growth and it exacerbates cancer invasion. Anyway, the biological effect of *SERPIND1* protein activity on different kinds of cancer is still largely unknown; particularly, it has never been studied in breast cancer.

Thus, for the first time, we assessed the relative expression of *SERPIND1* at mRNA and protein levels in the tissue and plasma of breast cancer patients, as well as evaluating the correlation between the relative expression of *SERPIND1* and the clinicopathological features. The results may help further studies aimed at a better understanding of the molecular mechanisms of the *SERPIND1* involvement in tumourigenesis, progression, and metastasis of breast cancer. The results can also pave the way for the development of new treatments for breast cancer patients.

Patients and Methods

Specimen preparation

Thirty-one Breast cancer tissues (BCT) and thirty-one non-tumoural adjacent tissues (NTAT) were collected by the Tumour Bank of the Cancer Institute (Imam Khomeini Hospital Complex, Tehran, Iran) regarding the rules of ethics in experimental and medical studies. The samples were taken from patients diagnosed with breast cancer and undergone surgery at Imam Khomeini Hospital Complex from 2008 to 2018. Tissue specimens were kept in sterilized cryo tube rack in a liquid nitrogen tank at −180°C since surgery until further analysis. The patients were in the age range of 32 to 81 years old with the mean age ± S. D of 54 ± 12. None of the patients had undergone radiotherapy, chemotherapy or any kinds of medication before the surgery. The specimens were fixed on slides and were stained using the hematoxylin & eosin (H&E) staining technique, and then were examined by a pathologist and confirmed as breast cancer. The clinicopathological factors including age, tumour size, types of metastasis, and the status of hormon receptors (HR), human epidermal growth factor receptor 2 (HER2), and p53 mutation were determined using the clinical and histopathological information available from the history of patients. Tumour stages were determined using the TNM Classification of Malignant Tumours of the Union for International Cancer Control, 8th edition[18] while tumour grade was determined by the pathologists using the WHO Classification of Tumours of the Breast, 4th edition[19].

Plasma Sample Preparation

Forty-three fasting venous blood samples were obtained from the patients who were referred to the Imam Khomeini Hospital Complex from 2008 to 2018 before surgery and any treatment. Patients were confirmed as breast cancer based on the results of the specific pathology tests, imaging, or biopsy before the initiation of any treatment modalities (patients aged from 31 to 81 years old with the mean age± S.D of 53. 67 ±12. 29. Also, venous blood samples were collected from forty-three healthy volunteers as the control group. The control group had no history of malignant diseases. They had not received any blood products within the last 3 years, and they were not in any inflammatory conditions at the time of sampling (individuals aged from 33 to 85 years old with the mean age± S.D of 20.50 ±14. 65. The samples were collected in EDTA-prepared tubes and immediately were centrifuged at 2000 × g for 10 min so that plasma was isolated, then they were kept in EP tubes (without enzymes) at −80°C since preparing until the analysis.

Tissue Homogenate Preparation

To prepare tissue homogenates, 100 mg of tissue slice was ground into fine powder using liquid nitrogen. Then 100 µL of phenylmethylsulfonyl fluoride (PMSF), the protease and phosphatase inhibitor, were added based on the protocol, followed by adding 1 mL of lysis buffer (contained 50 mM Tris-HCl pH:7. 4, 150 mM NaCl, 1mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Sodium deoxycholate). Samples were immediately centrifuged at 10000 × g for 5 min. The supernatant was carefully collected and kept in −180°C since preparing till the further analysis.

Total RNA Extraction

In the present study, we used the same amounts of tissue for RNA extraction to perform the normalization process with equal cell numbers. RNA extraction was performed using the GTC method (guanidinium thiocyanate-phenol-chloroform

extraction) with TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA, cat No. 15596026). This technique is an optimum mechanism for the single-step isolation of RNA developed by Chomcynski and Sacchi in 1987[20]. The process was as following: after homogenizing the samples using liquid nitrogen, 1 mL of TRIzol™ reagent was added to 100 mg of the homogenized tissue. The mixture was centrifuged and then 0.2 mL of chloroform was added. The RNA was precipitated from the upper phase using 0.5 mL of isopropanol. Electrophoresis (4% agarose gel) was used to control the quality of the extracted RNA and the spectrophotometry was performed using the *Nano Drop® ND-1000* UV-Vis Spectrophotometerto assess the purity and quantity of the extracted RNA. The Optical Density ratios of 1. 8-2 and 2-2. 2 were considered as the pure RNA at 260 nM *vs* 280 nM and 230 nM *vs* 260 nM, respectively.

cDNA Synthesis and Real-time PCR

The extracted RNA was converted to cDNA following the protocol of the kit (HelixCript™ *Thermo* Reverse Transcriptase, Nanoahelix, Yuseong-gu, South Korea, cat No. PT10K). All the steps of reverse transcription and PCR for the target and control groups were performed with the same accuracy, volume and in the same conditions of cDNA synthesis. The template RNA was introduced into the reverse transcription system with the same concentration of 1000 ng for all samples. PCR was performed using AMPLIQON Master Mix (Denmark, Cat No. A190303) based on the manufacturer instructions to evaluate the quality of the synthesized cDNA. Then the product underwent electrophoresis with 2% agarose gel and the process was confirmed. The synthesized cDNA was immediately stored at −20°C until the next use. The sequences of the primers of housekeeping gene, *Beta-actin*, and *SERPIND1* were, respectively as following:

Forward (*Beta-actin)*: 5′GATCAAGATCATTGCTCCTCCTG3′

Reverse (*Beta-actin)*: 5′CTAGAAGCATTTGCGGTGGAC3′

Forward (*SERPIND1*): 5′TGAAGTTGATGGGGATCAGG3′

Reverse (*SERPIND1*): 5′GACAGTGAAGCGGACTTGG3′

Real time PCR was performed using the RealQ Plus 2× Master Mix Green with low ROX™ kit (AMPLIQON, Denmark, Cat. No. A324406) and a thermal cycler (Exicycler™ 96 Real-Time Quantitative Thermal Block, BIONEER, Daedeok-gu, Republic of Korea). The reaction mixture with a final volume of 25 µL included:12.5 µL RealQ Plus 2× Master Mix Green (1X), 0.5 µL of forward primer (10 pmol/µL), 0.5 µL of reverse primer (10 pmol/µL), 2 µL of cDNA (100 ng/µL) and 9.5 µL Rnase-free water. The amplification reaction of the two-step Real-time PCR was performed double for each sample and the following regimen was used:

A 10 min cycle at 95°C, the main PCR cycle including a 15s at 95°C following a 60s at 60°C with 40 repetitions. The device software was used for calculation of the Ct (threshold cycle) values for the target and reference genes. The final Ct difference of 0.5 between the two repetitions was considered as acceptable and the mean Ct value was used as the final Ct. The liver tissue sample was considered as the positive control. The Livak method with the following formula was used to determine the Relative Expression (RQ) in both target and reference groups:

$$RQ = 2^{-\Delta\Delta Ct} \Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{Reference gene}})_{\text{tumour}} - (Ct_{\text{target gene}} - Ct_{\text{Reference gene}})_{\text{control}}$$

Assessment of plasma and tissue levels of *SERPIND1* protein in both groups of breast cancer patients and normal healthy individuals using ELISA

The *SERPIND1*protein concentration in the plasma samples, homogenates from BCT and NTAT were detected using the human HCII ELISA kit (Shanghai crystal Day Biotech Co., Ltd, Shanghai, China, Cat No: E1377Hu). The measurement steps were performed thoroughly according to the instructions of the kit manufacturer. The measurable concentration was in the range of 0.1-40 ng/mL and the analytical sensitivity was 0.051 ng/mL.

Data Statistical Analysis

Statistical analysis were performed using SPSS software (Version 20; SPSS Inc., Chicago, IL, USA) and the graphs were drawn using Graphpad Prism8 (Graphpad Software, La Jolla, California, USA, http://www. graphpad. com). The quantitative variables were initially evaluated for distribution and variance equality using the 1-sample K-S and Levene's test, respectively. The independent T-test and Mann-Whitney U Test were used to compare between two groups with normal and non-normal distribution, respectively. The one-way ANOVA with DunnettT3 post hoc test was used to compare between more than two groups with normal distribution and the Kruskal-Wallis H test was used to compare more than 2 groups with non-normal distribution. $P< 0.05$

was considered as the significance level and in the figures, *, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$ and ****, $P< 0.0001$ were considered.

Results

Evaluation of*SERPIND1* expression in the tumoural tissues and non-tumoural adjacent tissues

SERPIND1 expression was measured by qRT–PCR in 31 BCT and 31NTAT as control group. As shown in (Fig. 1A), SERPIND1 mRNA expression was significantly higher in BCT compared with NTAT ($t(60)= 1.89$, $P= 0.04$).

Correlation of the *SERPIND1* relative expression with the clinicopathological factors of Breast Cancer

As shown in Table 1, *SERPIND1*relative expression in BCT correlates significantly with the histological grade. The relative expression of *SERPIND1* in grade III Breast Cancer patients was significantly higher than patients with grade I (Table 2 & Fig. 1B). Besides, there was a significant correlation between the relative expression of *SERPIND1* and the cancer stage (Table 1). Information regarding the correlation directions in the different stages is summarized in (Table 2), where *SERPIND1* relative expression in the patients with stage II and stage IV breast cancer were significantly higher than the patients with stage I (Fig. 1C). Also, we found that the *SERPIND1* relative expression was significantly correlated with the tumour size (Table 1). The analysis showed that the *SERPIND1* relative expression was significantly higher in the tumours bigger than 5 cm compared to those smaller than 2 cm (Table 2). The relative expression of *SERPIND1* had no significant correlation with the age of the patients (Table 1).

Moreover, there was a significant positive correlation between the HER2 protein expression, the cancer grade (correlation coefficient= −0.574, P value= 0), stage (correlation coefficient= −0.628, P value= 0), and the presence of metastasis (correlation coefficient= −0.418, P value = 0.005). The status of p53 mutations was positively correlated with the cancer stage (correlation coefficient=0.732, P value= 0) and grade (correlation coefficient=0.489, P value= 0.001) and the presence of distant metastasis (correlation coefficient= −0.608, P value= 0). There was also a significant positive correlation between the ER and PR phenotypes with the cancer stage (correlation coefficient= 0.781, P value= 0) and grade (correlation coefficient= 0.632, P value= 0), lymph node metastasis (correlation coefficient= 0.602, P value= 0), and distant metastasis (correlation coefficient= −0.667, P value= 0).

Relationship between the *SERPIND1* relative expression in breast tumoural tissue and the invasive factors of the cancer

Table 3 summarizes the relationship between the *SERPIND1* relative expression in the BCT and the cancer invasion factors. According to our results, the relative expression of the *SERPIND1* was significantly higher in the patients suffering from metastatic cancer including distant metastasis, lymphatic invasion, lymph node invasion, perineural invasion, and vascular invasion, compared to the patients with non-metastatic

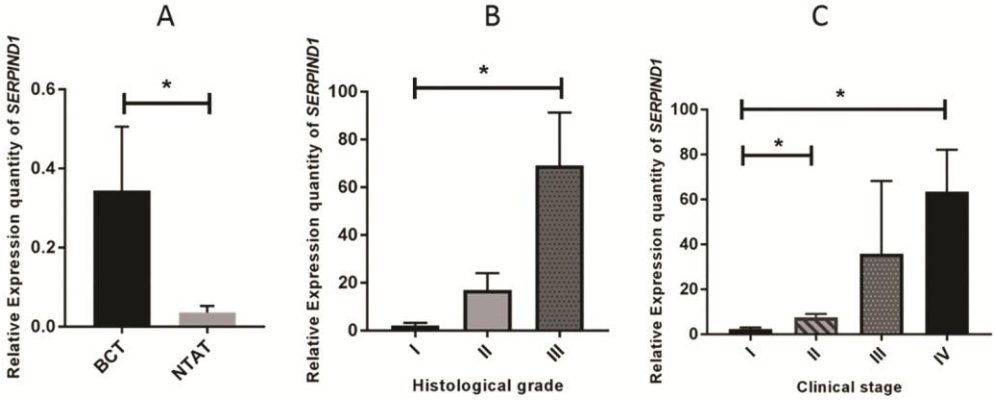

Fig. 1 — (A) Relative expression of *SERPIND1* in breast cancer tissue (BCT) (n=31) compared with non-tumoural adjacent tissue (NTAT) (n=31). The figure shows the quantitative values of expression in scale $2^{-\Delta Ct}$. As shown, the expression of *SERPIND1* mRNA is significantly higher in BCT than in NTAT ($P< 0.05$). The mean difference between two groups (± SEM) was calculated as −0.308 (± 0.162); (B) Comparison of relative tissue expression of *SERPIND1* in three histological grades of breast cancer; (grade I (n=6): well differentiation, grade II (n=15): moderate differentiation, grade III (n=9): poor differentiation). As shown, the relative expression of the *SERPIND1* mRNA in Grade III is significantly higher compared to Grade I ($P< 0.05$); and (C) Relative expression quantity of *SERPIND1* in association with four stages of breast cancer. The relative expression of *SERPIND1* in stage I (n=6) has significantly decreased compared to stage II (n=11) and stage IV (n=11) (for both $P< 0.05$).

Table 1 — Correlation between relative expression of SERPIND1 in breast cancer tissue and clinicopathological features.

		Relative expression of *SERPIND1*						
		N (31) (%)	Mean± Std. Deviation	Standard error	df1 (Between groups)	df2 (Within groups)	F	*P* value
Age(year)	30-40	4(13)	6.65±10.51	5.25	2	28	0.5	
	40-50	8(25.8)	28.07±40.03	14.15				0.5
	>50	19(61.2)	34.34±54. 20	12.43				
Grade	I	6(20)	2.19±2. 47	1.00	2	27	6.2	
	II	15(50)	16.96±27. 55	7.11				0.006*
	III	9(30)	69.18±66. 20	22. 06				
Stage	I	6(19.3)	2.32±1.57	0.64	3	27	4.5	
	II	11(35.5)	7.53±4.93	1.48				0.01*
	III	3(9.7)	35.84±55.97	32.31				
	IV	11(35.5)	63.58±61.50	18.54				
Tumour size	<2 cm	9(29)	5.48±6.69	2.23	2	28	3.591	
	2-5 cm	11(35.5)	21.54±41.49	12.51				0.04*
	>5 cm	11(35.5)	56.11±59.78	18.02				

Table 2 — Comparison of SERPIND1 relative expression in different grades and stages of breast cancer .

		Relative expression of *SERPIND1*		
Histology grade (A)	Histology grade (B)	Mean Differences(A-B)	Std. Error	*P* value
Grade I	Grade II	−14.77	7.18	0.15
	Grade III	−66.99	22.09	0.04*
Grade II	Grade III	−52.21	23. 18	0.13
Stage (A)	Stage (B)			
Stage I	Stage II	−5.20	1.62	0.03*
	Stage III	−33.52	32.32	0.84
	Stage IV	−61.25	18.55	0.04*
Stage II	Stage III	−28.31	32.35	0.90
	Stage IV	−56.05	18. 60	0.06
Stage III	Stage IV	−27.73	37.26	0.95
Tumour size(A)	Tumour size(B)			
<2 cm	2-5 cm	−16.06	12.70	0.52
	>5 cm	−50.62	18.16	0.04*
2-5 cm	>5 cm	−34.56	21.94	0.33

Table 3 — Correlation between relative expression of SERPIND1 in breast cancer tissue with metastasis, hormone receptor status (ER and PR), HER2 protein expression status, and p53 tumour suppressor protein mutation status (*Contd*.)

		Relative expression of *SERPIND1*				
		N (31) (%)	Mean± Std. Deviation	Df	t	*P* value
Distant Metastasis	Yes	11(35.5)	63. 58±61.50	11. 38	2.78	0.01*
	No	20(64.5)	10.21±21.70			
Lymphatic invasion	Yes	20(64.5)	42. 96±54.32	19. 37	3.188	0.005*
	No	11(35.5)	4. 04±3. 98			
Lymph node invasion	Yes	18(58.1)	43. 58±56.75	20.83	2.43	0.02*
	No	13(41.9)	9. 16±16.56			

(*Contd*.)

Table 3 — Correlation between relative expression of *SERPIND1* in breast cancer tissue with metastasis, hormone receptor status (ER and PR), HER2 protein expression status, and p53 tumour suppressor protein mutation status

		Relative expression of *SERPIND1*				
		N (31) (%)	Mean± Std. Deviation	Df	t	*P* value
Perineural invasion	Yes	12(38.7)	59.71±62.02	12.29	2.70	0.01*
	No	19(61.3)	9.84±18. 82			
Vascular invasion	Yes	19(61.3)	45.01±55.01	18.27	3.234	0.005*
	No	12(38.7)	4.04±3.79			
Necrosis presence	Yes	17(54.8)	34.53±51.62	29	0.693	0.4
	No	14(45.1)	22.61±42.29			
ER status	Positive	15(48.4)	10.75±25.05	20.90	−2.28	0.03*
	Negative	16(51.6)	46.39±56.78			
PR status	Positive	15(48.4)	10.75±25.05	20.90	−2.28	0.03*
	Negative	16(51.6)	46. 39±56.78			
HER-2	Positive	12(38.7)	50.36±64.77	13.24	1.76	0.1
	Negative	19(61.3)	15.75±25.84			
P53 status	Positive	15(48.4)	12.58±24.74	20.56	2.02	0.056
	Negative	16(51.6)	44.68±57.98			
Laterality	Left breast	12(40)	38.70±60.42	16.8	0.731	0.4
	Right breast	18(60)	24.35±37.97			

Table 4 — Descriptive statistics of *SERPIND1* protein concentration in plasma of breast cancer patients and healthy individuals. Mean concentration of *SERPIND1* protein in plasma is 8.27 ng/mL higher with standard error of 2.28 ng/ml in breast cancer patients compared to normal subjects

	Concentration of *SERPIND1* protein in plasma (ng/mL)						
	N	Mean	Median	Std. deviation	Mean Rank	Z	*P* value
Breast cancer patients	43	14.30	3.19	17.09	50.70	−2.67	0.008**
Healthy individuals	43	6.02	2.62	9.52	36.30		

cancer. The *SERPIND1* relative expression was also increased in patients with necrotic tumours compared to patients with non-necrotic tumours; however, the difference was not significant.

Relationship between the *SERPIND1* relative expression and the presence of hormonal receptors, HER2 protein expression, and the mutation status of the tumour suppressor protein p53

Table 3 summarizes the relationship between the *SERPIND1* relative expression in BCT and the presence of hormonal receptors, HER2 protein expression, and p53 mutation status. There was a significant increase in the *SERPIND1* relative expression in the HR-negative patients compared to HR-positive patients. Also, the *SERPIND1* relative expression was higher in the HER2-positive and p53-negative (having mutations in p53) patients than the HER2-neutral (without HER2 expression) and p53-positive (without p53 mutation) patients; However, the differences were not significant.

Evaluation of plasma *SERPIND1* protein levels in the healthy and cancer groups and the relationship between the *SERPIND1* protein levels and the clinicopathological features

As shown in Table 4, the plasma levels of *SERPIND1* protein were significantly higher in the breast cancer group compared to the control group (Fig. 2A). The analysis showed that the expression level of *SERPIND1*protein in plasma had a significant correlation with the cancer grade (Table 6). Our comparative pair analyses showed significant differences in *SERPIND1* protein levels between grade I and grade III patients (*P* value= 0.001) and also between grade II and grade III (*P* value= 0.009) (Fig. 2B). Also, the *SERPIND1* protein expression in the plasma of breast cancer patients had a significant correlation with the clinical stage of cancer (Table 6). Our results found significant differences in the *SERPIND1* protein expression between stage I and stage IV (*P* value= 0.02) and also between stage II and stage IV (*P* value= 0.005) (Fig. 2C). In addition, our results showed that plasma expression of *SERPIND1*

Table 5 — Descriptive Statistics of *SERPIND1* protein concentration in Plasma of Breast cancer patients in early stage of disease compared to healthy individuals. Mean concentration of plasma *SERPIND1* protein is 1.13 ng/mL higher with standard error of 2.64 in breast cancer patients compared to normal individuals, which was not statistically significant

	Concentration of *SERPIND1* protein in plasma (ng/mL)						
	N	Mean	Median	Std. deviation	Mean Rank	Z	*P* value
Breast cancer patients in Early Stages (stageI & II)	24	7.16	2.77	11.78	35.25	−0.39	0.6
Healthy individuals	43	6.02	2.62	9.52	33.30		

Table 6 — Correlation between mean plasma levels of *SERPIND1* protein in patients with breast cancer with clinicopathological features

		Plasma *SERPIND1* protein levels (ng/mL)						
		N(43) (%)	Mean	Std. Deviation	E standard error	Mean Rank	Df	*P* value
Age(year)	30-40	7(16. 3)	4. 66	3. 81	1. 44	17. 86	2	0.5
	40-50	11(25. 6)	14. 95	16. 43	4. 95	24. 73		
	>50	25(58. 1)	16. 70	19. 02	3. 80	21. 96		
Grade	I	10(23. 25)	3. 99	3. 18	1. 00	13. 40	2	0.001*[*]
	II	19(44. 18)	8. 40	11. 55	2. 65	18. 74		
	III	13(30.23)	31. 77	18. 13	5. 02	31. 77		
Stage	I	7(16. 27)	7. 88	13. 67	5. 17	15	3	0.002**
	II	17(39. 53)	6. 86	11. 36	2. 75	16. 06		
	III	7(16. 27)	19. 54	18. 03	6. 81	26. 29		
	IV	12(27. 9)	25. 51	19. 56	5. 64	32		
Tumour size	<2 cm	12(27. 9)	7. 14	10.77	3. 11	16. 50	2	0.1
	2-5 cm	13(30.23)	13. 79	17. 45	4. 80	21. 23		
	>5 cm	18(41. 9)	19. 43	19. 18	4. 52	26. 22		

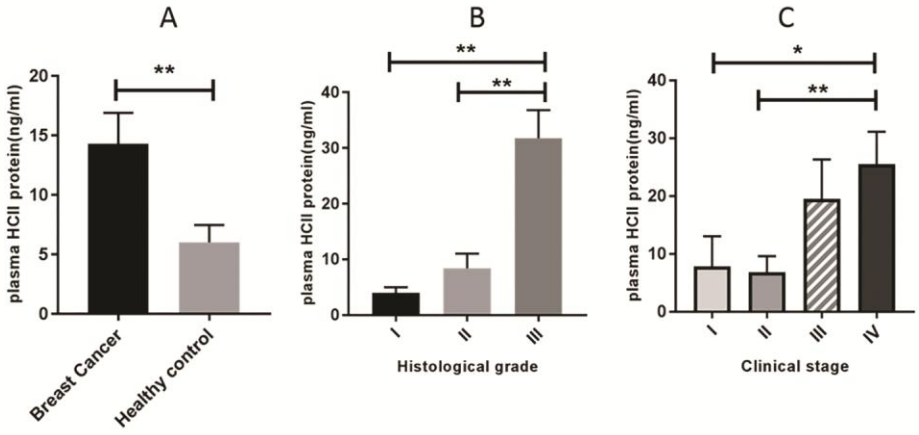

Fig. 2 — (A) The expression of *SERPIND1* protein in the plasma sample of breast cancer patients (n=43) compared to healthy control group (n=43). As shown in the figure, the plasma level of *SERPIND1* protein expression in breast cancer group is significantly increased compared to the control group ($P< 0.01$); (B) Comparison of plasma levels of *SERPIND1* protein in breast cancer patients at different histological grades. The figure shows that plasma expression of *SERPIND1* protein in breast cancer patients of grade III (n=13) is significantly increased compared to patients in grade I (n=10) and II (n=19) (for both $P< 0.01$); and (C) The expression of *SERPIND1* protein in plasma of breast cancer patients in four stages. Significant increase in plasma *SERPIND1* protein concentration in stage IV breast cancer patients (n=12) compared to stage I (n=7) ($P< 0.05$) and II (n=17) ($P< 0.01$)

protein in early-stage breast cancer groups (stage I and II) was not significantly different from healthy controls (Table 5). Finally, no significant correlation was found between the mean of *SERPIND1* protein concentration with the tumour size and age of patients (Table 6).

Relationship between the plasma concentration of *SERPIND1* protein and the invasion factors, the status of ER, PR, and HER2, and p53 mutation status

As shown in Table 7, the results showed that the mean of *SERPIND1* protein plasma levels was higher in the patients with distant metastasis, lymphatic

Table 7 — Correlation of plasma levels of *SERPIND1* protein in breast cancer patients with metastasis, hormone receptor status (ER and PR), HER2 protein expression status, and p53 protein mutation status

		Plasma *SERPIND1* protein levels (ng/mL)					
		N(43) (%)	Mean	Std. Deviation		Z	*P* value
Distant metastasis	Yes	12(27.9)	25.51	19.56	Mean Rank	−3.249	0.001**
	No	31(72.1)	9.95	14.11			
Lymphatic invasion	Yes	28(65.1)	19.80	18.97	32/00	−2.21	0.02*
	No	15(34.9)	4.02	2.91	18.13		
Lymphnode invasion	Yes	25(58. 1)	18.70	18.40	25.11	−2.36	0.01*
	No	18(41. 9)	8.17	13.26	16.20		
Vascular invasion	Yes	26(60.5)	19.34	19.28	25.84	−1.63	0.1
	No	17(39.5)	6.58	9.02	16.67		
Necrosis presence	Yes	21(48. 8)	17.80	18.65	24.54	−1.21	0.2
	No	22(51.2)	10.95	15.13	18.12		
ER status	Positive	23(53.5)	8.60	12.57	24.38	−2.508	0.001**
	Negative	20(46. 5)	20.85	19.46	19.73		
PR status	Positive	23(53.5)	8.60	12.57	17.52	−2.508	0.001**
	Negative	20(46.5)	20.85	19.46	27.15		
HER-2	Positive	18(41.9)	19.46	18.63	17.52	−2.11	0.03*
	Negative	25(58.1)	10.58	15.20	27.15		
P53 status	Positive	21(48.8)	9.61	14.27	26.78	−1.99	0.04*
	Negative	22(51.2)	18.77	18.64	18.56		

Table 8 — Descriptive statistics of *SERPIND1* protein concentration in breast cancer tissue homogenate compared to the non-tumoural adjacent tissue. Mean concentration of the protein is 0.62 ng/mL higher with standard error of 1.54 in breast cancer tissue in contrast to the margin of non-tumourous tissue; The difference is not statistically significant

	Concentration of *SERPIND1* protein in tissue hemogenate (ng/mL)					
	N	mean	Std. deviation	Mean Rank	Z	*P* value
Breast cancer tissue	31	4.50	7.13	31.69	−0.085	0.9
non-tumoural adjacent tissue	31	3.88	4.77	31.33		

invasion, and lymph node invasion compared to the healthy individuals. Also, the mean concentration of *SERPIND1* protein in the plasma from the HR-negative, HER2-positive, and p53-negative patients were significantly higher than the HR-positive, HER2-negative, and p53-positive patients.

Measurement of *SERPIND1*protein concentration in the breast tumoural tissue homogenates

The evaluation of *SERPIND1* protein concentration in the tissue homogenate samples obtained from the BCT and NTAT showed that the mean protein concentration in the tumoural homogenate was higher than the non-tumoural adjacent tissue, which was used as the control. However, the difference was not significant (Table 8).

Discussion

SERPIND1 protein tracing and its role in tumourigenesis and progression of a variety of cancers including B-Cell acute lymphoblastic leukemia[13], ovarian epithelial cancer[14] and lung cancer[15] have been studied. According to the results of these studies, the overexpression of *SERPIND1* appears to play an important role in tumour growth promotion, stimulation, and invasive behaviour increase of tumoural cells. However, the relationship between the expression of this gene and other cancer types including breast cancer has not been investigated.

At the present, clinically early cancer screening method based on traditional protein tumor marker of serum such as CA and CEA has low sensitivity, low accuracy and poor specificity. So new specific tumor marker of serum are always needed[16]. In the present study, we found that there is a significant increase in *SERPIND1* expression at mRNA and protein levels in the BCT compared to the NTAT and in the plasma from the breast cancer patients compared to the healthy volunteers, respectively. Moreover, we showed that the mean of *SERPIND1* protein concentration was higher in the BCT than the NTAT of the same patients by assessing the *SERPIND1*

protein concentration in tissue homogenates; however, the increase was not significant, maybe due to the small sample size. According to the study analyses, the *SERPIND1* expression at both mRNA and protein levels in the breast cancer patients showed that there is a significant correlation between the clinicopathological features including grade, stage, and size of the tumour. Consequently, the increase in the disease grade leads to an increase in the *SERPIND1* expression in the tumoural tissue and plasma of the patients. The increase was highly significant and pronounced in patients with grade III breast cancer compared to grade I. Also, our assessments showed that the *SERPIND1* expression in tissue and plasma of the patients increased as the disease stage progressed, and the gene had the highest expression in stage IV. However, in our study, plasma levels of *SERPIND1* protein were not able to differentiate the breast cancer patients in the early stages (stages I and II) from healthy individuals. In other words, if *SERPIND1* protein is used as a screening marker, it will increase the overall number of people diagnosed with breast cancer, but may not be able to detect the disease in the early stages of tumour formation. Moreover, our results suggested that larger tumours express the *SERPIND1* gene at significantly higher levels than the smaller tumours. It has been reported that there was a significant overexpression of *SERPIND1* in epithelial ovarian cancer, and the patients with higher *SERPIND1* expression levels in the ovarian cancer tissue had a poor prognosis, which was in line with our results. Evaluating 113 patients with ovarian epithelial cancer showed that there is an increased expression of *SERPIND1* in stages III and IV compared to stages I and II. This finding was consistent with our results as well. They also reported that the decrease in the tumoural cell differentiation caused an increase in the expression level of *SERPIND1*; however, it was not significant. This result was not consistent with the results of the present study[14,17-20].

We found that the *SERPIND1* expressions, at both mRNA and protein levels, were significantly higher in the breast cancer patients with distant metastasis, lymphatic invasion, and lymph node metastasis compared to those whose disease was not extended (non-metastatic). Incompatible with our results, a group found that the patients suffering from ovarian epithelial cancer with lymph node metastasis had an increasing trend of *SERPIND1* expression compared to those with no lymph node metastasis; however, they did not find this increase as significant[14]. Previous studies found that the *SERPIND1* expression increased the cell mobility in Non-small-cell lung carcinoma(NSCLC) and also the cancer invasion and metastasis, both *in vitro* and *in vivo*[15], which was consistent with the results of our study. The study found that Ras-related C3 botulinum toxin substrate 1(Rac1) and Cell division control protein 42 (Cdc42) are the *SERPIND1* downstream effectors in a PI3k-dependent manner, and heparin can inhibit the *SERPIND1* effects on cell migration, invasion, and metastasis. They stated that the anti-metastatic effect of heparin was more pronounced on the cancer cells with overexpression of *SERPIND1*[15]. In other words, maybe the expression levels of *SERPIND1* in the tissue and plasma can be used as a predictive factor for the glycosaminogly can therapy with agents such as heparin. Further clinical trials are needed to confirm this hypothesis.

A study has reported that serpins including *SERPIND1* have a role in breast cancer metastasis to the brain[17]. It was also shown that the overexpression of *SERPIND1* in the malignant breast tissue had a significant association with the metastasis development, which was in line with our results. This effect may be due to several mechanisms. It has also been confirmed that Rac1 and Cdc42 activities in lung cancer cells showed an increase following the treatment with exogenous *SERPIND1* protein, suggesting that Rac1 and Cdc42 could be the filopodium formation effectors through *SERPIND1* protein and result in an increase in the migration of lung cancer cells[15]. This effect may explain the metastatic potential of the lung cancer cells with *SERPIND1* overexpression; because the filopodium number is closely correlated with the invasive phenotype in cancer cells[21,22]. Extensive studies are needed to understand the association of filopodium number in breast cancer cells with *SERPIND1* overexpression. Since a pronounced and significant increase was found in the expression of the RHO proteins of Rac1 and Cdc42 in the BCT compared to the NTAT of the same patients[22, 23],these results can provide a general explanation for the involvement of RHO GTPases in human carcinogenesis and the consequent effect of *SERPIND1* overexpression on the invasion of breast cancer in the present study. Considering all findings, it is possible that the *SERPIND1* overexpression in BCT had also additive effects on the proliferation, invasion and metastatic

potential of the breast cancer cells in our study by altering the regulation of the signaling pathways mentioned, increasing the filopodium number and RHO protein expression, as well as changing the cell cycle. However, these interpretations may provide the basis for further studies in this area aimed to clarify the possible role of this factor as a metastasis enhancer in breast cancer. Moreover, it was found that *SERPIND1* caused increases in the proliferation, migration, invasion, the transition from G1 phase into S phase of the cell cycle, and the epithelial-mesenchymal transition (EMT) in the ovarian cancer cells while decreasing the apoptosis of these cells through increasing the phosphorylation of PI3K and AKT. Also, it was reported that PI3K was possibly a downstream effect or of *SERPIND1*[24-26]. Various studies reported that EMT played a key role in the initiation of metastasis and increasing the migration and invasion capacity of the cancers including breast cancer [27-29]. According to the results of the present study, it is also possible that the overexpression of *SERPIND1* was associated with the PI3K activation and EMT increase in breast cancer cells, thereby increasing the invasion potential of them. Further studies can discover new molecular markers to predict disease recurrence and prognosis as well as new targets for gene therapy and medication development by creating cell lines with *SERPIND1* overexpression, clarifying the regulatory mechanisms associated with this gene, and a better understanding of the regulatory mechanisms associated with EMT in breast cancer.

In the present study, all samples had HR-positive or HR-negative phenotypes. By gene expression analysis, we found that the relative expression of the *SERPIND1* gene showed significant increases in tumoural tissue and plasma of HR-negative breast cancer patients compared to HR-positive individuals. According to the reports, the increase in the number of hormonal receptors in tumoural cells increased the response to endocrine therapy while decreasing the disease recurrence and mortality rates; besides, women with receptor-positive phenotypes had a better prognosis including lower tumour proliferation rates and histologic evidence of higher tumour differentiation[30-32]. Our study was in line with these interpretations, showing higher expression levels of *SERPIND1* were in HR-negative patients. This expression had a positive correlation with grade and stage of the disease and tumour size, probably suggesting a poor prognosis in the ER-negative and PR-negative patients with *SERPIND1* overexpression. Moreover, we showed that the lack of hormonal receptors had a positive correlation with the disease grade, disease stage, axillary lymph node metastasis, and distant clinical metastasis. These findings support the results above. In conclusion, it can be said that the *SERPIND1* overexpression, which correlates with the lack of HR expressions in cancer cells, results in higher tumour growth and higher spread of it. Further studies are necessary on the underlying mechanisms through which *SERPIND1* leads to increased tumour growth and its association with the HR status.

Our findings showed that the patients with HER2 expression in cancer cells had significantly higher plasma levels of *SERPIND1*protein than the patients who lacked HER2 expression. Overexpression of HER2 resulted in an increased response to the stromal growth factors and oncogenic transitions. It has also been reported that HER2 is associated with malignancy development, a significant reduction in the survival rate, and poor prognosis of breast cancer[33-35]. Also, we found a positive correlation between the disease stage and grade with the plasma expression of HER2 protein; since the HER2-positive breast cancer is more invasive, we should consider that the increased levels of *SERPIND1*protein in the plasma of these patients can be an indicator of adverse prognosis, more invasive tumour, and a consequent higher likelihood of metastasis in breast cancer patients, which can be helpful in the targeted treatment of the patients. However, more studies are needed to clearly understand the relationship between *SERPIND1* expression and the expression level of the HER2 in breast cancer patients.

Our analysis also indicated that patients with mutations in p53 protein had significantly higher plasma levels of *SERPIND1*protein compared to the patients without p53 mutations. Various studies have reported that p53 mutations possibly were associated with more invasive disease, distant metastasis, and less total survival rate in breast cancer patients[36]. Our study showed that the increase in tumour size and cancer stage increases the possibility of p53 mutations. In agreement with this result, other studies reported that mutations in this protein were significantly more prevalent in T3 tumours (larger than 5 cm)[37]. In the present study, overexpression of *SERPIND1* was found in breast cancer patients with p53 mutations. Since these changes in p53 usually occur in the advanced stages of breast cancer, more

extensive studies are needed to confirm that *SERPIND1* expression could be associated with p53 mutation and the metastatic potential of the cancer cells.

The present study was the first to investigate the association between *SERPIND1* and breast cancer tumourigenesis and the more invasive behaviour of breast tumours. It has shed a light on the potential use of *SERPIND1* protein as a plasma tumour marker or targeted therapy. However, further assessments are needed to elucidate the underlying mechanisms.

Conclusion

The evidence provided by the present study showed that *SERPIND1* might be a novel metastasis-enhancing factor in breast cancer, a strong predictor for identifying the patients with poor prognosis, and a marker for disease recurrence estimation. At the same time, these results may help the development of new agents for breast cancer targeted therapy. Further studies on *SERPIND1* and its role in the molecular processes involved in tumourigenesis, cell growth, and metastasis in breast cancer may elucidate the underlying mechanisms behind the rise of biologically malignant features of breast cancer through *SERPIND1*.

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Conflicts of interest

All authors declare no conflict of interest.

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