

Identified Survivin Expression Profile as a Biomarker in Breast Cancer, A Cross-Sectional Study

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Abstract

Background: Defects in cell signaling are common and play a crucial role in the development and progression of breast cancer. These defects can include alterations in cell-cell communication, uncontrolled cell growth, and resistance to programmed cell death (apoptosis). Survivin is a protein that plays a role in the regulation of apoptosis and has been observed to be overexpressed in breast cancer. To evaluate the potential of Survivin gene expression as a biomarker in the breast cancer. **Materials and Methods:** By using Real-Time PCR, expression of Survivin, Caspase-3, Caspase-8, Caspase-9 and TNF- α were detected in 84 female patients after surgery, 42 malignant and 42 benign samples. About one gram from inside tumor and another one gram beside the tumor was taken to isolate mRNA by RT-PCR which detects the expression of Survivin, Caspase-3, Caspase-8, Caspase-9 and TNF- α inside the tumor tissue and beside them. Besides tumor tissue, 84 blood samples were collected from patients to detect the expression of Survivin and other caspases, in addition to that, 50 blood samples were collected from women without evidence of breast cancer as a control group. All the samples were collected from patients admitted to Al-Basrah Teaching Hospital during the period between March 2022 and October 2024. **Results:** Expression of Survivin showed a higher level in malignant tissue compared with benign samples. Inside malignant tissue showed high expression in Survivin compared to tissue beside the malignant site. Caspases-3 and Caspase-9 which are related to the apoptosis process, showed decreased expression inside malignant samples where Survivin increased. Caspase-8 also showed slightly increased and TNF- α was elevated inside malignant tissue compared with tissue beside the malignant site. Expression of Survivin in blood samples of patient with malignant tumor showed increased level compared to blood sample of benign tumor and with control blood samples. **Conclusions:** The study showed that Survivin expression in malignant tissue was elevated compared to benign tumours. The variation in Survivin was evident also in blood samples with declining gradient from malignant, to benign and normal controls.

Keywords: Breast cancer, Survivin, biomarkers

Asian Pac J Cancer Nursing, 301-308

Submission Date: 07/20/2026

Acceptance Date: 09/04/2026

Introduction

Breast cancer is a significant public health issue, being the most frequently diagnosed malignancy among women globally and associated with high rates of morbidity and mortality [1]. Breast cancer occurs at a rate of about 145 cases per 100,000 women annually, with a mortality rate of 33 per 100,000 women each year [2]. Early diagnosis is highly beneficial, as early detection, along with advancements in breast cancer treatment, can help

to improve survival [3].

The interplay between genetic predisposition and environmental factors, including obesity, lack of physical activity, alcohol use, hormone replacement therapy, and menstrual history, are linked to the risk and progression of breast cancer [4]. Hence, carcinogenesis occurs due to an imbalance between cell proliferation and apoptosis [5]. Apoptosis is a controlled process of cell death that plays

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an essential role in development and maintaining balance in the body [6]. Several genes play roles in regulating apoptotic signaling pathways, which are functioning as either pro-apoptotic or anti-apoptotic factors. Among the proteins that suppress apoptosis, survivin is considered a crucial component [5].

Survivin, an essential component of the inhibitor of the apoptosis protein (IAP) family, is distinguished by its remarkable features. It is the smallest protein within this family [6]. The Survivin gene is situated in the 17q25 region of the human genome, Survivin is strikingly overexpressed in almost all cancer types, yet its presence is minimal in most normal adult tissues [7]. Its distinctive structural and functional properties make it a compelling subject of interest in cancer research. It is critical to various cellular processes, significantly affecting tumor cell survival and metastasis. Moreover, growing evidence points to its wider role in preserving immune system balance [8]. Survivin is expressed in multiple splicing variants, each carrying distinct functional roles. These variants arise from the alternative splicing of the Survivin gene and have been extensively studied for their potential importance in cancer biology and treatment strategies. For example, variants like Survivin-3B and Survivin-ΔEx3 have been associated with changes in apoptotic regulation and tumor progression [9].

In contrast, Survivin-2B has demonstrated potential as a tumor suppressor, with its expression being linked to better patient outcomes in specific cancer types [10]. Researchers analyzed Survivin expression in 60 different tumor types, including lung, esophageal, breast, ovarian, stomach, and prostate cancers. These studies identified the highest Survivin levels in breast and lung cancers, while kidney cancer showed comparatively lower expression [11].

Survivin unusually shows high expression in diverse tumor types and its strong link to poor disease outcomes have positioned it as a promising diagnostic biomarker [12- 14].

To summarize the role of Survivin inside the cell, we have to study the proteins that contribute with Survivin, like Caspase 3; Caspase 8; Caspase9. When the cell has a defect, apoptosis will be induced either by extrinsic or intrinsic pathway. Extrinsic pathway is induced by TNF-alpha, while intrinsic pathway is activated by death stimulus like UV or Gamma radiation and chemotherapy. In an extrinsic pathway, TNF-alpha activates Caspase-8 followed by activating Caspase-3 which lead to DNA fragmentation and cell death [12]. Here, Survivin showed blockage of Caspases-3 and stop its action in apoptosis when the cycle is finished. On the other hand, intrinsic pathways lead to activating Caspase-9 either directly or through mitochondrial enzymes then activating Caspase-3 to switch on the apoptosis pathway. The role of Survivin is to block the activation of mitochondrial enzymes and inhibit Caspase-9 to ensure finished the cycle when it was complete. During cancer growth, the activity of Survivin will be disrupted and showed phosphorylated of Caspases in a long time which stopes apoptosis and enhances replication of the cell even if it is faulty [13, 14].

The present study aims at evaluating the potential of Survivin gene expression as a biomarker in breast cancer.

Materials and Methods

Study population and Sampling

The study was a cross-sectional study, conducted between March 2022 and October 2024 in Basrah Teaching Hospital. Biopsy samples and blood were collected from 84 female patients who were admitted to the surgical ward with benign or malignant breast mass. In addition, other 50 blood samples were taken from females who clinically do not suffer from breast tumor as control samples.

Excisional biopsy for benign tumor and mastectomy or breast conserving surgery for malignant tumor were performed according to the result of Fine Needle Aspiration (FNA). The inclusion criteria included patients with breast tumor diagnosis and had been histopathologically confirmed and did not receive any treatment in the form of chemotherapy or radiotherapy.

Tissue extraction

After the diagnosis of breast tumors (malignant and benign cases), the patients were admitted to the hospital. and during surgery (lumpectomy, breast conserving surgery or mastectomy), a biopsy of about 1gram of breast tumor was taken from each sample of both benign and malignant cases, as well as from tissue located 2cm away from the tumor, which is named as a beside tumor tissue, and considered as non- tumor tissue. The tissue specimens were preserved in a container containing normal saline and then transported to the laboratory of Al-Zahraa College of Medicine. The tissues were washed by phosphate buffer saline (PBS) twice, then cut into small pieces and washed again, tissues were mashed up in sterile distill water and crushed with a sterile mortar with 2ml Nuclease-Free Water to get homogenize liquid. 0.75 ml of TRIzol™ LS Reagent kit (Solarbio, Shanghai, China) was added to 0.25 ml of sample suspension then homogenize the sample by pipetting up and down several times to lyse the tissue.

Blood mRNA extraction

According to (Dongsheng biotech, N1121) mRNA blood mini kit protocol, blood was collected in tubes with Ethylenediaminetetraacetic acid (EDTA) anticoagulant to prevent clotting and stabilize the sample. A volume of 200 µl blood was mixed with 800 µl of phenol and guanidinium isothiocyanate solution (TRI solution Invitrogen) for 10 minutes at 25°C. Then, 250µl of chloroform solution was added and the mixture was incubated at room temperature with mixing for 5 minutes.

Centrifugation of tissue and blood samples for subsequent mRNA extraction

The solution was centrifuged at 10,000 g for 20min at 4°C. The supernatant layer was collected in a new clean tube with 500µl of isopropanol and mixed well, then incubated at 25°C for 15 minutes. The solution then centrifuged at 10,000g for 20 minutes at 4°C to precipitate

mRNA which then collected and washed with ethanol 70% and dissolved with 40µl free RNase water.

mRNA extraction and cDNA synthesis

Reverse transcription quantitative polymerase chain reaction (RT- qPCR) was used to detect the level of the markers through their mRNA in the samples. mRNA concentration was confirmed by nanodrop spectrophotometer using the ratio optical density OD 260/280. The extracted mRNA was used directly to avoid storage under -80C°. Referenced primers of Survivin were performed from the literature [15] to avoid making mistakes of primer designing. The markers; Survivin, Caspase 3, Caspase 8, Caspase 9, TNF-α and GAPDH primers were obtained from Promega, USA, (Table 1). Regarding cDNA synthesis, 1 µg of extracted cellular mRNA was used for cDNA synthesis by oligonucleotide primers, which were designed to detect Survivin by RT-PCR, product length: 338 bp, shown in Table 1.

The RT-PCR procedure was performed using 1X SYBR Green PCR Master Mix [16], and started with reverse transcription at 55 °C for 60 minutes, followed by inactivation of the transcriptase at 95 °C for 8 minutes. Amplification was then carried out for 40 cycles of denaturation at 95 °C for 20 seconds, annealing at 55 °C for 20 seconds, and extension at 72 °C for 50 seconds [17].

Detection of Survivin level in samples

Reverse transcription PCR of total mRNA was performed in accordance with instructions provided by Reverse Transcription System A3500 Promega, USAa. [18].

Equation 1

$\Delta CT (\text{Sample}) = \Delta CT (\text{target gene}) - \Delta CT (\text{reference gene}).$

Equation 2

Relative expression = (CT Survivin; Caspase-3; Caspase -8; TNF-α), – (CT GAPDH)

Statistical analysis

For the purpose of statistical analysis, SPSS software, version 26, was used. The data were presented in terms of mean and standard deviation; and to investigate the statistical significance of differences in means, T-test was applied. A p-value of ≤0.05 was considered as significant result.

Results

Distribution of patients according to age and type of tumor (M=malignant, B= benign)

The study was conducted on 84 patients presented with breast mass. Patients' age ranged from 16 to 75 years old. The patients were categorized into six groups according to age. Samples were classified as 42 (50%) malignant tumor and 42 (50%) benign tumors as shown in Table 2. The result also showed that the highest proportion of malignant cases was in age group 56-66-year (11, 26.2%), followed by age group 46-55-years with 10 cases (23.8%). However, only 2 and 3 samples (4.76%, 7.14%) were collected in age group 26-35 and 18-25- years respectively. The benign tumors were distributed in the same age groups as follows: the age group 46-55-year (12, 28.57%), 56-66-years (10, 23.8%) and 36-45- year (10, 23.8%). The details are shown in Table 2.

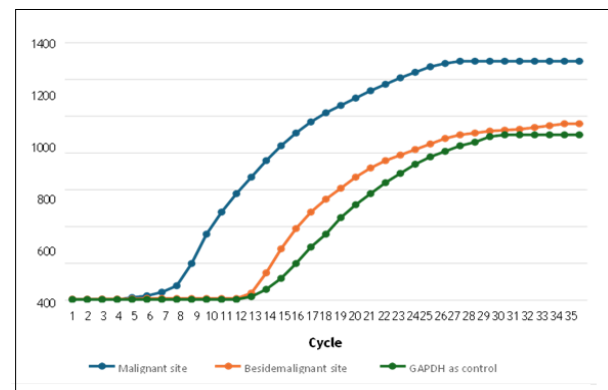


Figure 1. Amplification Plot of Sample of Survivin in Malignant Site and Beside Malignant Tissue Compared to GAPDH.

Table 1. Primers of the Markers Details and Their References.

Markers	Sequence	Ann. Temp.	No. bp	Ref.
Surviv.	5'ATGGGTGCCCCGACGTTGC3' 3'GTCGACCGACGGTACCTAACT5'	58	338	19
Casp. 3	5'CTC GGT CTG GTA CAG ATG TCG ATG3' 3'GTT TAA CCC GGG TAA GAA TGT GCA5'	60	540	20
Casp. 8	5'GGGAAGTGTTTTCACAGGTT3' 3'TAAGGCGTTTCCTTCGTTCTT5'	60	411	21
Casp. 9	5'-ATGGACGAAGCGGATCGGCGGC-3' 5'-TTATGATGTTTTAAAGAAAAGTTTTTTC-3	58	388	22
TNF-α	5'CGAGTGACAAGCCTGTAGC3' 3'TACACGAGGAGTGGGTGTGG5'	57	454	23
GAPDH	5' ACGGGGTCACCCACACTGTGC3' 3'GTAGCAGGTGGCGTTTACGAAGATC5'	60	392	23

Table 2. Histopathological Results of Samples According to Age Group (M= malignant; B= benign).

Age group	Type				Total		Control	
	M	%	B	%	No.	%	No.	%
16-25	3	7.14	4	9.52	7	8.33	6	12
26-35	2	4.76	5	11.90	7	8.33	8	16
36-45	7	16.66	10	23.80	17	20.23	7	14
46-55	10	23.80	12	28.57	22	26.19	12	24
56-65	11	26.2	10	23.80	21	25.00	13	26
66-75	9	21.43	1	2.38	10	11.90	4	8
Total	42	100	42	100	84	100	50	100

Table 3. Cycle Threshold (Ct) Value of Survivin in Malignant Tissue Samples.

Age group	Ct value of Survivin in Malignant site and beside the malignant site of tissue		
	Survivin Malignant Site	Survivin Beside Malignant site	GAPDH
16-25	9.70	14.50	15.89
26-35	9.50	14.36	15.75
36-45	9.35	14.13	15.63
46-55	9.22	14.21	15.56
56-65	9.22	14.09	15.59
66-75	9.21	14.11	15.23
The Average	9.36	14.23	15.60

Estimating expression of Survivin by RT-PCR

Value of Cycle Threshold (Ct) of Survivin in malignant tissue was tested inside and beside the malignant tissue site and compared with Ct value of the cellular protein called Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The Δ Ct of samples is shown in (Table 3). Ct value of Survivin in tumor site of malignant tissue increased by an average of 1.5 times from the tissue

beside malignant site. The result showed that Survivin in malignant site appeared in cycle 9 while beside the malignant site showed in cycle 14 related to GAPDH which appeared during cycle 15. (Table 3; Figure 1).

Expression of mRNA level of Survivin in malignant and benign samples

The result of measuring expression of Survivin in malignant and benign samples by RT-PCR showed that there are significant differences between the expression level of mRNA of Survivin in malignant against benign samples. The highest expression was in the age group 56-65 years, which recorded 18.89 folds more related to mRNA of the housekeeping gene GAPDH. The lowest expression folds of Survivin were shown in the age group 46-55 years 11.3 folds. While the expression in benign sample was no more 2.49 in age group 26-35 and the lowest expression was 1.19 in age group 36-45 years. The result also determined the difference between samples that they got from inside and besides the samples. In malignant, the average of Survivin expression inside malignant tissue increased 14.2 folds compared with GAPDH, while it was only 1.42 folds beside the malignant site. The highest expression of the samples beside the

malignant site was 1.8 folds and the lowest was 1.02-fold in the age groups 56-65 and 16-25-years respectively. In benign sample, the average changes in sample of inside and beside the tumor site was 1.89 and 1.54 respectively (Table 4).

Differences in the expression of Survivin related to GAPDH between malignant and benign biopsies as presented in Table 5, were found statistically significant ($p=0.0001$).

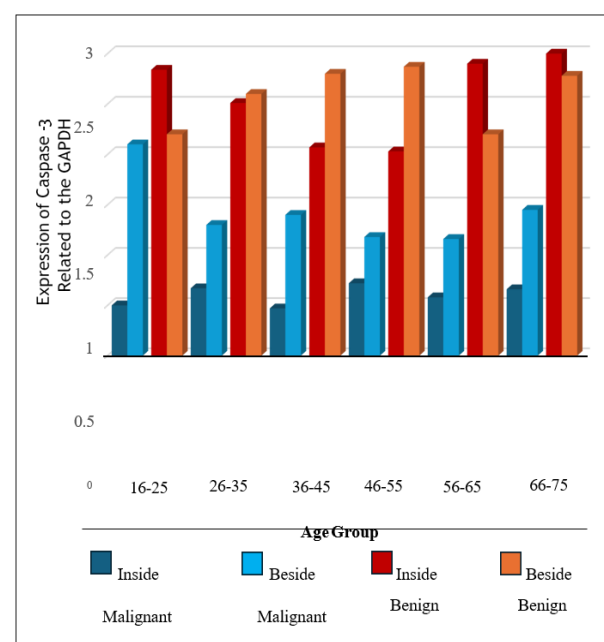


Figure 2. Expression of Caspase-3 Inside and Beside the Malignant and Benign Samples During Ages.

Table 4. Expression of Survivin in Malignant and Benign, Inside the Tumor and Beside It.

Age	Expression of Survivin related to GAPDH			
	Malignant		Benign	
	Inside	Beside	Inside	Beside
16-25	13.9	1.02	2.46	2
26-35	11.79	1.6	2.49	1.8
36-45	14.5	1.3	1.19	1.76
46-55	11.3	1.41	1.68	1.18
56-65	18.89	1.8	1.74	1.1
66-75	14.82	1.4	1.82	1.4
Average	14.2	1.42	1.89	1.54

Table 5. Differences in the Expression of Survivin Related to GAPDH Between Malignant and Benign Biopsies.

Marker expression	Malignancy	N	Mean	SD	T	P-value*
Expression of Survivin related to GAPDH	Malignant	12	10.0592	5.06617	5.680	0.0001
	Benign	12	1.7183	0.45708		

Differences in the expression of Survivin related to GAPDH in malignant biopsies, were found statistically significant when the sites of biopsy (inside and beside) were compared together (Table 6).

Expression of mRNA level of Caspase-3 in malignant and benign samples

The expression results of the mRNA of Caspase-3 showed decreases in malignant sites in all age groups compared with the housekeeping protein GAPDH. The expression inside the malignant showed less than beside the malignant site, and also less than that of benign samples, which showed no difference in inside and beside benign tumor tissue. (Figure 2).

Table 7 shows that there was significant ($p=0.0001$) statistical difference, in the expression of Caspase-3 related to GAPDH, between malignant and benign tumors. The mean expression in malignancy (1.0158) is significantly lower than expression in benign tumors (2.5667).

When the site of tumor was tested for the expression of Caspase-3 related to GAPDH, Table 8, it was found that in malignancy, the expression inside tumor (0.6) was significantly ($p=0.0001$) lower than the expression beside the tumor (1.4317). However, no such significance was noted in benign tumors ($p=0.939$) (Table 8).

Expression of mRNA level of Caspase-8 in malignant and benign samples

Expression of Caspase-8 showed an average increase of about 6 folds more than the expression of GAPDH in inside malignant tissue compared with tissue beside the malignant site, while benign tissue showed no significant increase in tissue compared with benign tissue. (Supplementary Table 1).

Regarding the expression of Caspase-8 related to GAPDH, it is shown in (Supplementary Table 2) that, also, there was a significant statistical difference ($p=0.003$) in this expression between malignant and benign tumors. Here, the expression was significantly higher in malignant tumors (3.7925 versus 06).

In Table (Supplementary Table 3), it can be noted that the expression of Caspase-8 related to GAPDH in malignant tumors is significantly ($p=0.0001$) higher in biopsies taken from the inside of the tumor (6.4867 versus 1.0983). Such a difference is absent in benign tumors ($p=0.471$).

Caspase-9 in malignant and benign samples

Similar to the result of Caspase-3, the expression of Caspase-9 showed suppressed in malignant tissue. Expression value of Caspase-9 inside malignant tumor showed less than those in samples of beside malignant tumor as well as benign tissues (Figure 3).

Similar findings were found ($p=0.011$) regarding expression of Caspase-9 related to GAPDH in malignant versus benign tumors (Supplementary Table 4).

Expression of Caspase-9 related to GAPDH

Also, significant differences were documented in Table (Supplementary Table 5). In malignant ($p=0.0001$) and in benign (0.001) tumors, statistically significant differences were found in the expression between inside and beside biopsies

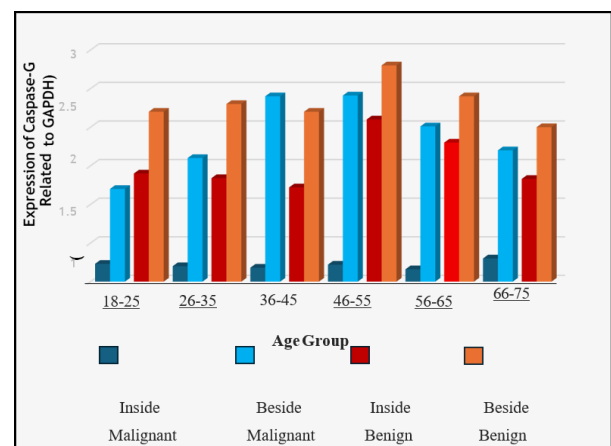


Figure 3. Expression of Caspase -9 in Inside and Beside the Malignant and Benign Samples During Ages.

Table 6. Differences in the Expression of Survivin Related to GAPDH in Malignant Biopsies and in Benign Biopsies According to the Site

Malignancy	Site	N	Mean	SD	t	P-value
Malignant	Expression of Survivin related to GAPDH Inside	6	14.2	2.71214	5.184	0.0001
	Beside	6	4.9183	2.821		
Benign	Expression of Survivin related to GAPDH Inside	6	1.8967	0.49923	1.411	0.189
	Beside	6	1.54	0.36617		

Table 7. Differences in the Expression of Caspase-3 Related to GAPDH between Malignant and Benign Samples

	Malignancy	N	Mean	SD	t	P-value
Expression of Caspase-3 related to GAPDH	Malignant	12	1.0158	0.49797	-8.798-	0.0001
	Benign	12	2.5667	0.35336		

Table 8. Differences in the Expression of Caspase-3 related to GAPDH in Malignant Biopsies and in Benign Biopsies According to the Site

Benign	Expression of Caspase-3 related to GAPDH	Inside	6	2.5583	0.42687	-.078-	0.939
		Beside	6	2.575	0.30383		
Benign	Expression of Caspase-3 related to GAPDH	Inside	6	2.5583	0.42687	-.078-	0.939
		Beside	6	2.575	0.30383		

TNF- α in malignant and benign samples

TNF- α showed a highly increased inside malignant and benign tumor in average of 26 and 18 folds respectively related to GAPDH. The expression of TNF- α inside malignant tissue was increased 5 times more than in tissue beside the malignant tissue and about 4 times more in benign

Unlike the previous findings, the expression of TNF- α related to GAPDH does not statistically significantly differ between malignant and benign tumors ($p=0.259$), (Supplementary Table 6).

Expression of mRNA level of TNF Alpha

However, in (Supplementary Table 7), there is a significant difference in expression of TNF- α between biopsies from inside and those from beside the tumor, whether in malignant ($p=0.0001$) or in benign ($p=0.0001$) tumors.

Survivin in blood samples of patients and controls

The Ct-value of Survivin in blood samples was analyzed and revealed that the curve of Survivin in patient

blood appeared early before the control samples and GAPDH as shown in sample of the Ct value (Figure 4). *Expression of Survivin in blood of malignant, benign, and control cases*

The expression value of survivin in blood of malignant cases indicated an increase in about 6 times comparing with the expression in blood of the control and benign cases. Among the age groups, the age 26-35- years showed slightly more increased compare with the average (Figure 5).

Expression of mRNA level of Survivin in blood of malignant, benign, and control cases

The results show highly significant differences in Survivin expression between malignant and both benign/control groups ($p < 0.0001$), whereas no significant difference was observed between benign and control groups ($p=0.26$).

RT-PCR amplification of Caspases in blood samples

The Ct value of Caspases revealed that the mRNA of Caspases 3, 8 and 9 were not successfully amplified during RT-PCR reaction of blood samples. The reaction was repeated 5 times with no clear results. On the contrary, GAPDH showed regular curve as shown in selected sample (Figure 6).

Discussion

In the current study the samples, which were divided into 42 of malignant and 42 of benign cases, concentrated in three age groups 36-45, 46-55, 56-65 for both malignant and benign cases. In other study the range of affected women were concentrated in 51-60 age group [19]. The expression of Survivin showed elevated levels inside malignant tissue compared with samples beside the malignant tissue site. The results are consistent with the

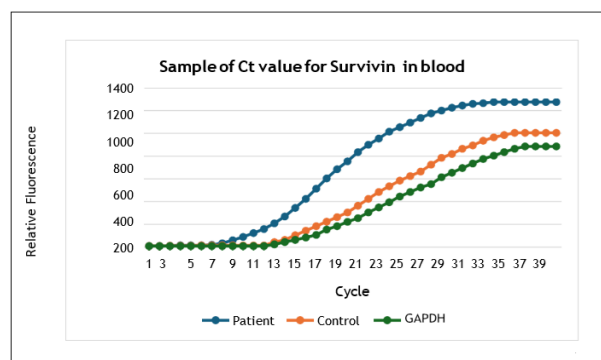


Figure 4. Amplification Plot of Sample of Survivin in Blood of Patient and Control Sample Compares to GAPDH.

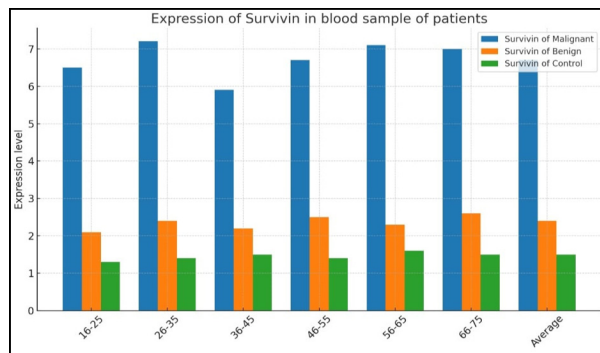


Figure 5. Expression of Survivin in Blood Samples of Malignant, Benign and Control Cases.

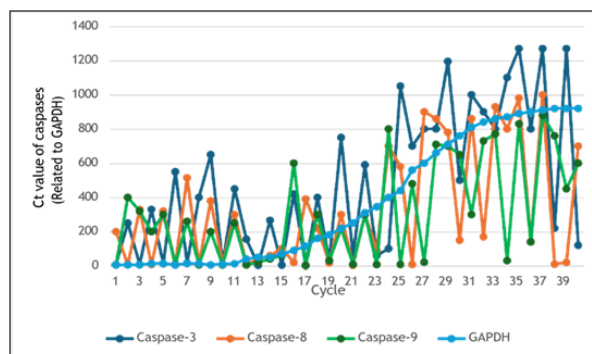


Figure 6. Example of Disrupted Amplification Plot of Sample of Caspases in Blood Compares to GAPDH Regular Amplification Plot

findings of a study published in 2023 [20]. High expression of Survivin in the samples during age 56-65-year in about 17 times more than benign samples may be related to high-grade cancer tissues, the same results was confirmed by other studies [20, 21]. The highly expression of Survivin in malignant tumor compared with benign tumor in our study is also consistent with the results of another study published in 2010 [22]. The result of the current study has shown that while the expression of Survivin is increased the expression of Caspases 3 and 9 were suppressed inside the breast cancer malignant tissue compared with beside the malignant tissue and benign samples. The role of Survivin in inhibiting of apoptosis requires suppressing of Caspases through shutting off their enzymatic action or mainly downregulate Caspase-3 and 9 [23, 24]. The mechanism by which Survivin inhibits the action of Caspases in cancer cells is represented by phosphorylation of Thr34 in Survivin which facilitate binding of Survivin to Caspases 3 and 9 and deactivate them [7]. The result also showed that Caspase-8 was expressed in about 5 to 6 folds more inside malignant tissue than beside malignant and benign tissue. This finding is supported by the result of another study. A recent study confirmed that some group of caspases like 6, 7, 8, 10, has not been shown to be inhibited during the activity of Survivin, but some of them like Caspase 8 is activated during induction when TNF- α is increased [25]. The result of this study highlighted that the TNF- α also appeared to be activated and increased in its expression in about 5 times more inside malignant tissue compared

with beside the malignant site, which is compatible with other studies [26].

Detection of Survivin expression in patients' blood has been one of the aims of this study. The results detected high expression level of Survivin in the peripheral blood samples of the malignant cases compared with the control group and with samples taken from patients with benign tumors, especially since the results showed that the expression of Survivin in blood samples of patient with malignant cases is more in 3-4 times than in blood samples of benign and control cases. The expression of Survivin in blood of malignant cases showed no significant differences between age groups. This result is compatible with most of the researches which showed that Survivin expression is increased in blood sample of more than 90% of malignant breast tumors compared to patient with benign breast tumor and control women [21, 27].

The study failed to detect Caspases in blood samples. The mRNA of Caspases 3, 8 and 9 were not amplified in blood of patient and control by RT-PCR. The Ct blot showed an irregular and unreliable noisy blot which refers to an amplification problem. This may be due to diluted and oscillating concentration levels of Caspases, and they have limit half-lives, and complex signaling interactions [28]. The Ct curve of GAPDH was shown regularly and confirmed that there was no troubleshooting in the process of RT-PCR. In conclusions, Survivin expression is correlated with breast cancer occurrence with variation in its level in malignant tissues and blood. Caspases 3 and 9 were shown to be disrupted in malignant tissue compared with benign tissue. While the expression of Caspase 8 was identified to be slightly increased in malignant tissue.

Acknowledgments

Statement of Transparency and Principles

- The authors declare no conflict of interest.
- The study was approved by the Research Ethics Committee of the authors' affiliated institution.
- The study data are available upon reasonable request.
- All authors contributed to the implementation of this research.

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