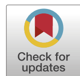
Heliyon

Received:
13 November 2018
Revised:
14 February 2019
Accepted:
14 March 2019

Cite as: Hien Minh Nguyen, Minh Quang Dao, Huyen Thi La. Performance of *survivin* mRNA as a biomarker for breast cancer among Vietnamese women. Heliyon 5 (2019) e01371.
doi: 10.1016/j.heliyon.2019.e01371



Performance of *survivin* mRNA as a biomarker for breast cancer among Vietnamese women

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Abstract

Objective: This study aimed to perform the reverse-transcription polymerase chain reaction (RT-PCR) to express the *survivin* mRNA among patients with breast cancer in Vietnam and identify some potential associated clinical and pathological factors.
Methods: Peripheral blood (PB) samples and tissues on 43 patients with breast cancer and 21 patients with fibroids were obtained. The Real-time RT-PCR and gene sequencing techniques were employed to detect survivin gene in breast cancer cell lines and cancer tissues.
Results: *Survivin* mRNA transcription was detected in 32/43 (74,4%) of breast cancer tissues and 19/43 (44,2%) of PB samples of breast cancer patients, while it was detected in only 14,3 % fibrosis tissues and 0% in the blood of fibrosis patients. *Survivin* mRNA on the peripheral blood of breast cancer patients increased with tumor size, and stage of cancer ($p < 0.05$). In terms of breast cancer tissue, no difference was found in the rate of *survivin* mRNA expression in according to age, distant metastasis, lymph node, stages of cancer, and histopathology ($p > 0.05$).
Conclusions: Results provide the initial evidence of the expression of survivin mRNA in breast cancer patients in Vietnam, suggesting the role of *survivin* mRNA in breast cancer molecular pathology.

Keyword: Oncology

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1. Introduction

Breast cancer is one of the most common types of carcinoma among women in global settings. Recent estimate indicated that there were more than 2.4 million and 523,000 new breast cancer incidents and breast cancer-related deaths in 2015, and 69% of breast cancer burden were observed in low and middle-income countries (Fitzmaurice et al., 2017). Individualized medicine is vital for breast cancer patients given its benefit in providing tailored and appropriate treatment based on personal and disease's characteristics. This strategy requires insights of various histopathological and molecular factors such as tumor size, lymph node conditions and human epidermal growth factor receptor 2 (HER2), which help to understand the initiation and progression of breast cancer in each patient (Foekens et al., 2008; Olopade et al., 2008; Ross et al., 2009; Xu et al., 2014; Zografos and Roukos, 2011). However, despite progress in the personalized treatment, more than 30% of patients suffered breast cancer recurrence (Park et al., 2010; Thiery et al., 2006). Therefore, it is important to investigate a new marker that can provide an accurate stratification among patients with breast cancer, and support the optimization of current therapies for breast cancer (Xu et al., 2014).

Survivin, a multifunctional protein, is a member of the inhibitors of apoptosis proteins (IAP) family, composing 142 amino acid with the length of 16.5 kDa and locating on the chromosome 17q25 (Jha et al., 2012). The roles of survivin in the proliferation of cancer cells as well as poor prognosis and treatment outcomes have been documented (Altieri, 2003; Jha et al., 2012; Ryan et al., 2006). Thus, the expression of survivin has been concerned as a target for cancer treatment (Altieri, 2008; Guha and Altieri, 2009). In recent years, some molecular techniques have been used to understand the *survivin* mRNA expression in breast cancer patients including reverse transcriptase polymerase chain reaction (RT-PCR), immunohistochemistry and ELISA (Jha et al., 2012). However, when several prior evidence revealed a positive association between the expression of survivin and positive outcomes, other studies showed a contradict finding (Kennedy et al., 2003; Span et al., 2004; Yakirevich et al., 2012). Further studies should be required to validate the use of survivin expression in detecting the progression of breast cancer.

This study aimed to perform the RT-PCR to express the *survivin* mRNA among patients with breast cancer in Vietnam and identify some potential associated clinical and pathological factors. Data of this study would partly contribute to understanding the prognostic importance of survivin in women suffering from breast cancer in Vietnam.

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2. Material and methods

2.1. Study design and sampling

A total of 43 patients with confirmed breast cancer in all stages were recruited at the time of diagnosis at the K Hospital − the largest oncology hospital in Vietnam. They were excluded if they had tumors in other organs, were treated by any therapies and did not accept to participate. Also, twenty-one fibroids patients in the same hospital were also invited to participate in the study. A convenient sampling technique was used to recruit patients.

2.2. Blood samples

Five milliliters of peripheral blood in both breast cancer patients and fibroids patients were obtained and stored in ethylenediaminetetraacetic acid (EDTA). The blood samples were centrifuged 4000 revolutions per minute in 20 minutes. After that, the plasma was removed, and the white blood cells were collected to enrich the cells, including breast cancer cells. Samples were stored at 4 °C and processed immediately after being drawn.

2.3. Tissue samples

Tissue samples were obtained about 20−30 mg in similar patients who took the blood, after having surgery to remove the tumor. Samples were then stored in sterile vials at -80 °C until RNA separation.

2.4. Breast cancer cells

MCF7, BT474, KPL4, and MDA-MB231 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Invitrogen) + 10% FBS (Invitrogen) + 1% P/S (Invitrogen) + 2mM L-Glutamine. These cells were counted before the experiment.

2.5. RT-PCR procedure

Tumor cells in the blood were obtained by centrifuging blood samples with 4000 revolutions per minute (rpm) for 20 minutes. This technique can be used to enrich the nucleus in breast cancer cells. Cells in breast cancer tissues were taken after the tumor removal surgery. Samples were stored at 4 °C and sent immediately to the laboratory for total RNA extraction. Total RNA from breast cancer cells in the peripheral blood and tissues were processed using Mini Kit (Quiagen Kit Rneasy, Germany). The purity and concentration of total RNA were checked by using RNA absorption spectrum on NanoDrope -1000. The purity depends on the ratio

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of OD260nm/OD280nm, and the ratio ranging from 1.7 to 2.0 was acceptable for considering purity.

cDNA creation (RT-PCR) was done using the Invitrogen™ SuperScript™ II Reverse Transcriptase with up to1 pg of total RNA. Because the total RNA extracted from the tissues and blood samples were not similar, it was necessary to adjust the process from total RNA to cDNA to ensure that the imports for Real-time PCR had equivalent total RNA (about 100 ng). First, we used about 100 ng of total RNA and mixed with RNase-free water to reach 6 μl per tube. One μl Reaction mix and 1 μl Random hexamers were also added at 65 °C in 5 minutes; then, this combination was put on ice in 1 minute. Finally, 10 μl buffer and 2 μl enzyme were added to reach 20 μl per tube.

Synthesized cDNA with the following thermal cycles: 25 °C/10 min; 50 °C/50 minutes; 85 °C/5 minutes. The cDNA product was stored at -20 °C (according to the manufacturer's guideline - Invitrogen, USA). RT-PCR was performed with primers: *survivin, GAPDH*. A standard curve was built with breast cancer cells that had a certain number of cells (2×10^4). PCR reaction with cDNA was synthesized from sample and breast cancer cells, which was checked by survivin primers in Table 1.

2.6. PCR and sequencing

PCR reaction from cDNA was synthesized in samples and breast cancer cell lines using survivin and GAPDH primers. PCR product was checked by the electrophoresis with 1% agarose. Several samples were sequenced to confirm that the cloning genes were survivin genes.

2.7. Real-time PCR

Real-time PCR with survivin primers was conducted using the Roche's SYBR Green kit. Copies obtained from PCR products were computed as follows:

$$X\ (g)/\mu l\ DNA/[\text{length RNA} \times 2 \times 340] \times 6.022 \times 10^{23} = Y\ \text{copies}/\mu l$$

where: 340 is the molecular weight of a nucleotide

Table 1. Sequences of Primers used in this study.

Primer		Tm (°C)	length (bp)
GAPDH F	5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′	65.3	307
GAPDH R	5′- AGC CTT CTC CAT GGT GGT GAA GAC-3′	67	
*Survivin*F	5′-AGA ACT GGC CCT TCT TGG AGG-3′	63,3	170
*Survivin*R	5′-CTT TTT ATG TTC CTC TAT GGG GTC-3′	61,8	

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6022×10^{23} is the number of molecules in one mole of the substrate.

The number of original cDNA = Number of copies obtained from PCR/2n (n is the number of PCR cycles).

Standard curves were developed by estimating from diluting this amount of cDNA in each tube at the rate 10/100/1.000/10.000. In the SYBR Green, the amplified response could be seen through the emitted fluorescence signal, which attached to the double-stranded DNA from the start to the ends of the response, by using a camera system that can track the fluorescence signal in each tube. The results were applied to construct the standard curves and then were used for calculating the cDNA copies.

2.8. Statistical analysis

Data were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL). Chi-squared test was used to identify the differences of the survivin (+) expression in various groups. A p-value of less than 0.05 was considered statistical significance.

2.9. Ethical consideration

The study protocol was approved by the Institutional Review Board of the Ministry of Health and Hanoi Medical University (3018/QD-BYT). All patients were obtained informed consent before the experiment.

2.10. Data availability

The data of this study belongs to Thanh Nhan Hospital. Contact corresponding author for further request.

3. Results

Fig. 1 shows that, after cloning *Survivin* cDNA by PCR in four breast cancer cell lines, MDA-MB231, KPL4, MCF7 cell lines were found to be positive, while the BT474 cell line was negative. The expressions of survivin cDNA in blood peripheral and breast cancer tissue were similar.

Standard curves were developed using 20,000 MCF7 cell lines. According to the formula, the number of cDNA copies of survivin generated from breast cancer cell lines MCF7 was 10^5 copies. Based on this standard, it is possible to calculate the number of copies of *survivin* mRNA (Fig. 2).

Standard curves were made by measuring crossing point (CP) values (Table 2) at six reaction tubes. Tube survivin cDNA 1x was generated from 20.000 MCF7 cells,

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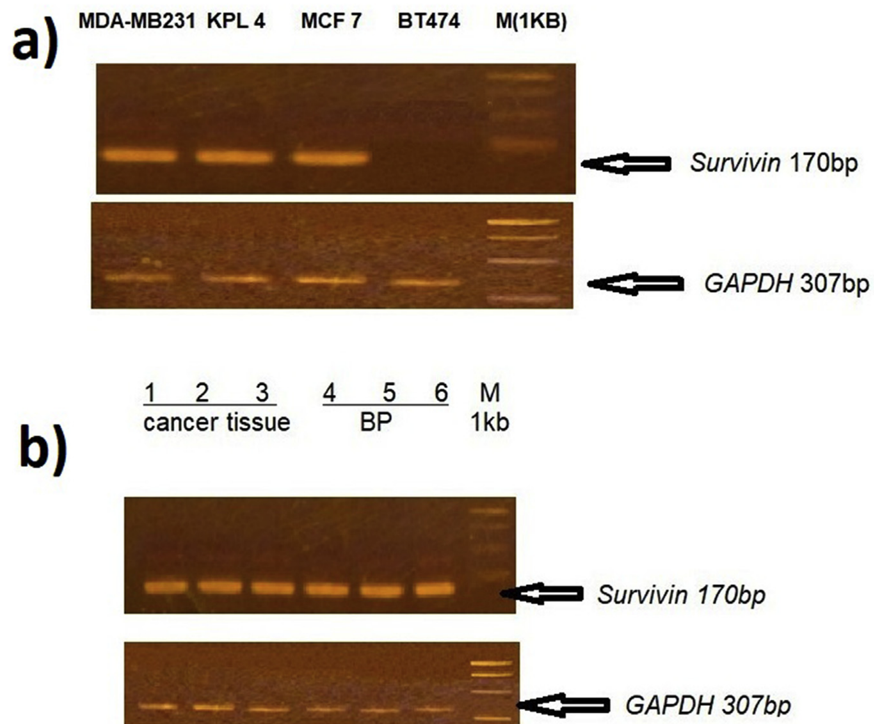

Fig. 1. PCR Electrophoresis image of Survivin cDNA and GAPDH. a) In breast cancer cell lines; b) In breast cancer tissue and PB.

which was equivalent to 10^5 copies. At a dilution of 100 times, which equaled to 200 breast cancer cells, the copies were detected with the CP at 26.14 and the number of copies at 1.08E4. Less CP value had a higher number of cDNA copies.

Table 3 shows that Survivin mRNA transcription was detected in 74.4% of breast cancer tissues and 44.2% of PB among breast cancer patients, while it was detected in only 14.3% fibrosis tissues and 0% in the blood of fibrosis patients.

Table 4 reveals that Survivin mRNA on the PB of breast cancer patients increased with tumor size and stage of cancer ($p < 0.05$). We did not find any differences in *Survivin* mRNA regarding the characteristics of breast cancer tissues.

4. Discussion

Survivin is the multifunction protein that has been considered potential candidates for the anti-cancer therapies due to its roles in proliferating cancer cells, inhibiting apoptosis and promote angiogenesis (Duffy et al., 2007). This study provided an insight of survivin expression in tissues and PB among breast cancer patients, thus might imply several clinical implications for improving the efficacy of current cancer treatment.

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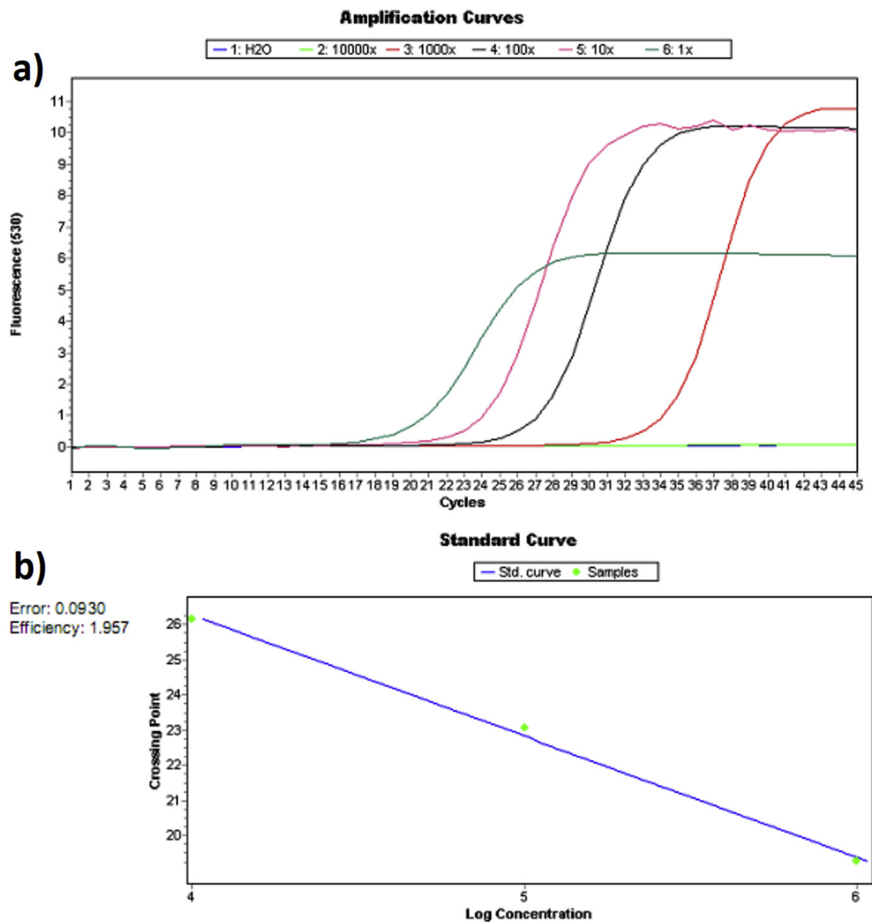

Fig. 2. Realtime PCR for Survivin cDNA to determine standard curve using MCF7 cells: a) Amplification Curves; b) Standard Curve.

In this study, the *survivin* mRNA expression was found in most of the breast cancer cases (74.4%). According to Spain et al., the rate of *survivin* mRNA expression among breast cancer patients was from 69.2 to 93.8% and varied in accordance to the stage of the disease. In our study, the rate of patients with stage

Table 2. Real-time PCR *Survivin* cDNA to determine the detection level in MCF7 cells.

Pos	Name	Type	CP	Concentration	Standard
1	H2O	Unknown			
2	10000x	Unknown			
3	1000x	Unknown	33.19	[9.51E1]	
4	100x	Standard	26.14	1.08E4	1.00E4
5	10x	Standard	23.05	8.58E4	1.00E5
6	1x	Standard	19.28	1.08E6	1.00E6

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Table 3. Survivin mRNA transcription in study.

Samples	n	survivin mRNA (+)
Breast cancer tissue	43	32/43 (74.4%)
Fibrosis tissue	21	3/21 (14.3%)
Peripheral blood in breast cancer patient	43	19/43 (44.2%)
Peripheral blood in fibrosis patients	21	0/21 (0%)

I of cancer having *survivin* mRNA was 50%, while this rate among those in phase III and IV was 87.5%. This finding was in line with a study of Li et al., which revealed that 85% breast cancer tissues had survivin mRNA expression, and they did not find any associations between survivin mRNA expression with age, histopathology, and tumor size (Li et al., 2012). A study of Span

Table 4. Survivin expression in breast cancer tissue and blood according to different characteristics.

Characteristics	No. of cases (n)	Survivin (+) in breast cancer tissues			Survivin (+) in peripheral blood of breast cancer patients		
		n	%	p-value	n	%	p-value
Age (year)							
≤50	21	16	76.2	0.7	6	28.6	0.051
>50	22	16	72.7		13	59.1	
Tumor size							
T1	10	5	50.0	0.1	2	20.0	**0.03**
T2	17	13	76.5		6	35.5	
T3 and T4	16	14	87.5		11	68.8	
Distant metastasis							
M0	38	28	73.7	0.76	15	39.5	0.08
M1	5	4	80.0		4	80.0	
Lymph node							
No	13	8	61.5	0.2	3	23.1	0.06
Yes	30	24	80.0		16	53.3	
Stage of cancer							
I	8	4	50.0	0.13	2	25.0	**0.04**
II	19	14	73.7		6	31.6	
III and IV	16	14	87.5		11	68.8	
Histopathology type							
Ductal	29	24	82.8	0.15	16	55.2	0.1
Lobular	8	4	50.0		2	25.5	
Mucous	6	4	66.7		4	66.7	
CA15-3 (U/ml)							
≤32	33	26	78.8	0.2	16	48.5	0.9
>32	10	6	60.0		3	30.0	

Bold values indicates <0.05.

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et al. in 275 breast cancer samples indicated that the expression of survivin mRNA in tissues was not associated with tumor size ($p = 0.46$), and lymph node metastasis ($p = 0.62$). According to Yamashita et al., using RT-PCT technique, the survivin expression was only related to tumor size, while it was not associated with other factors such as age, menopause, tumor size, histological characteristics, vascular endothelium, estrogen receptor (ER), and progesterone receptor (PR) (Yamashita et al., 2007). Despite differences in the reporting rates, these studies had a consensus that the expression of survivin mRNA could be found at a relatively high rate in breast cancer tissues in the early stage.

The *survivin* mRNA expression was found in 19 PB samples (44.2%), which was similar to other studies. A study by Yie SM et al. found that the detection rate of *survivin* mRNA in the PB was 50.7% (Yie et al., 2006). Moreover, the prevalence of *survivin* mRNA expression in the PB among patients with phase I, II, III and IV breast cancer patients was 25%; 31.6%; and 68.8%, respectively. Yamashita et al. in their study with 76 breast cancer concluded that *survivin* mRNA expression among patients in phase I, phase II and phase III was 16.1%; 33.3%; 88.8%, showing the relationship between the survivin expression in the PB and the stage of cancer (Shin-Ichi Yamashita, 2007). Moreover, these authors suggested that *survivin* mRNA in the blood was also associated with tumor size and lymph node metastases (Shin-Ichi Yamashita, 2007). In this study, we found that tumor size had a significant association with the survivin expression, while no relationship was found between age, histological characteristics, increased CA15-3 and lymph node conditions (Shin-Ichi Yamashita, 2007).

The strength of this study included the use of both PB and tissue samples of breast cancer patients for detecting the expression of *survivin* mRNA at the molecular level. The result would be useful to develop detection procedures in clinical settings. However, a small number of samples utilized in this study might limit the representativeness of our results, as well as could lead to false negative.

In summary, the results of the study provide the initial evidence of the expression of *survivin* mRNA in breast cancer patients in Vietnam, suggesting the role of *survivin* mRNA in breast cancer molecular pathology.

Declarations

Author contribution statement

Hien Minh Nguyen, Minh Quang Dao, Huyen Thi La: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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 https://doi.org/10.1016/j.heliyon.2019.e01371
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