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The role of exosomal survivin in the diagnosis of breast cancer

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ABSTRACT

Breast cancer is the leading cause of death in women across the globe. The aim of this study is to compare exosomal survivin levels in breast cancer patients and healthy population. It is also to investigate the relationship between clinicopathological parameters of breast cancer and exosomal survivin levels. Patients who were diagnosed with histopathologically confirmed breast cancer and healthy volunteers were included in the study. Serum samples from patients and healthy volunteers were stored at -80°C. Exosomes were isolated from these serum samples. The integrity of the exosomes was disrupted. Survivin levels were measured by Enzyme Linked Immuno Sorbent Assay. Data on clinicopathological parameters were obtained from patient files and compared statistically.

1. Introduction

Globally, breast cancer is a major public health concern among women. Breast cancer is the second most common cause of cancer-related deaths in women.[1,2] Advanced diagnostic and therapeutic methods and increased awareness for breast cancer in the society led to an increase in the number of patients diagnosed at an early stage. Early detection allows for implementation of effective treatments that could result in lower morbidity and mortality for the patient in comparison to considerably difficult therapies given at a later stage. Early diagnosis of patients, determining which patients could benefit from neoadjuvant or adjuvant therapy and assessment of the risk of recurrence on a patient basis are important factors that can help prolong survival of these patients. As a generic term, extracellular vesicles (EV) include all membrane vesicles secreted into the extracellular space.[3] The terms ectosomes, shedding vesicles, microvesicles and microparticles refer to large vesicles generally ranging from 150-1000 nm in diameter, which originate by direct outward budding of the plasma membrane. In contrast, the term exosomes refer to smaller vesicles with a diameter ranging between 30 and 100 nm; they are multivesicular bodies (MVBs) that are formed intracellularly and secreted into the extracellular space through fusion with the plasma membrane.[4] It has been shown that extracellular vesicles do not only carry cytosol and residual membrane proteins, but also play a role in the immune system modulation and interaction of the immune system and cancer cells.[5,6] Exosomes act

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as non-cellular communication vehicles carrying signaling molecules including cellular proteins.[7] After exosomes are released from cells of origin; they carry a variety of cargo proteins and genetic information to regulate conditions such as growth and differentiation. In the last decade, exosomes have received much attention globally because of these unique roles. Their role in cancer progression is of particular interest. Exosomes have been isolated from cell culture medium and various body fluids.[8,9] So they can be easily obtained. This property of exosomes has led to the thought that they can serve as a biomarker in the early detection of cancer. Survivin is a member of the inhibitor of apoptosis (IAP) family. Survivin has a role in the inhibition of cell death, regulation of mitosis, control of cell cycle checkpoints and adaptation to unfavorable conditions.[10,11] The abnormally high expression of survivin in cancer cells with little expression in most normal cells makes it an attractive anticancer target. Survivin has been found in the nucleus, cytosol, mitochondria and more recently in the extracellular space.[12] In immunocompromised animals, mitochondrial survivin accelerates tumor growth and abolishes tumor cell apoptosis in vivo, whereas its extracellular form has the ability to reenter cancer cells, inducing tumor cell proliferation, apoptosis resistance and increased invasion.[13] In light of the mounting evidence for the involvement of exosomes in the carcinogenesis, we aimed to determine the role of exosomes and exosomal survivin in breast cancer.

2. Materials and Methods

In recent years, there has been a growing interest in the importance of RNA and other molecules carried by exosomes and other extracellular molecules. These molecules may play a key role in the detection of circulating biological molecules. Until now, conventional methods involving purification of exosomes for RNA isolation have been time consuming and produced inconsistent results due to the use of ultracentrifugation. The first commercial exosome isolation kit (ExoQuick) developed by the System Biosciences (SBI) allows isolation of exosomes from serum, plasma or ascites fluid without the need for an ultracentrifugation step. In electron microscopy studies, exosomes isolated with ExoQuick have been shown to be similar to exosomes isolated using ultracentrifugation. These exosomes are also active in several functional assays.[14,15]

2.1. Patient selection and sampling

Approval for the present study was obtained from the Ethics Committee of Gaziantep University Faculty of Medicine on 11.01.2016 as per decision no. 2016/16 and the study was conducted on the principles set forth in the Declaration of Helsinki. All patients were informed about the study and gave written consent. The study enrolled patients with a histopathologically confirmed diagnosis of invasive ductal breast cancer who were followed at Medical Park Gaziantep Hospital Medical Oncology Clinic between 2014 and 2017 and healthy volunteers. Patient files were reviewed retrospectively to obtain demographic and clinical data including age, gender and routine laboratory workup. Breast cancer patients who were under the age of 18 years and patients with insufficient hepatic and renal reserves were excluded. Leftover blood samples from previous sampling obtained from the patients just before initiation of first-line systemic chemotherapy for routine follow-up were collected in a prospective manner. Blood samples were centrifuged at 1000 rpm for 15 minutes within 1 hour after collection. Sera obtained were aliquoted in microtubes and immediately frozen at -80 °C.

2.2. Isolation of exosomes

First, sera stored at -80^{o}C were thawed on ice. Centrifugation was performed at 3000 g for 15 minutes to allow precipitation of cells and particulates. Following centrifugation, an appropriate volume of the supernatant was transferred into a sterile tube. Then, 63 μl ExoQuick exosome precipitation solution was added to 250 μl serum. The tubes were inverted to mix the solution with serum and incubated at +4^{o}C for 30 minutes. At this point, the tubes were not moved any longer to allow the solution to reach the bottom of the tube. Following incubation, the sample was spun at 1500 g for 30 minutes at room temperature. The pellet resulting from centrifugation contains exosomes. The supernatant was discarded and spun at 1500 g for 5 minutes to precipitate residual material. All liquid phase was discarded taking care not to disturb the pellet. Subsequently, the pellet was dissolved in 300 μl 1X PBS for the next step.

2.3. Disruption of the membrane integrity of the exosomes and quantitation of exosomal survivin

Exosome membrane integrity was disrupted by adding 100 μl lysis buffer (GF-1, Vivantis, Malaysia) devoid of ELISA inhibitor to 300 μl mix. The standard solutions of 2.000 pg/mL, 1.000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL and 31.2 pg/mL were prepared by diluting the stock standard. 100 μl of each standard solution and a blank were added into the appropriate wells (1 to 8 wells) and 100 μl samples were added into the other wells. The plate was covered with a plate sealer and incubated at 37^{o}C for 1 hour. Then, the remaining liquid was removed without rinsing the plate. 100 μl Detection Reagent A (reconstituted by 1:100 dilution of the concentrate solution of Detection Reagent A) was added into each well and incubated at 37^{o}C for 1 hour. Following incubation, the solution was aspirated and washed with 350 μl wash solution, and allowed to sit for 2 minutes. Then, the remaining liquid was removed from

the wells completely. Washing was repeated for a total of 3 times. After the last wash, any remaining wash buffer was aspirated (Wash solution was prepared by diluting 20 mL concentrate with 580 mL ddH_2O). Then, 100 μl Detection Reagent B was added to each well (Detection Reagent B preparation: same as Detection Reagent A) and incubated for 30 minutes at 37^{o}C. The wash process was repeated for a total of 5 times. After the washing step, 90 μl Substrate solution was added to each well. The plate was covered with a new sealer and incubated for 15 minutes at 37^{o}C. The liquid turned blue by the addition of Substrate solution. The plate was protected from light during this step. Following incubation, 50 μl Stop solution was added to each well. The liquid turned yellow by the addition of Stop solution. The liquid was mixed by tapping the side of the plate if color change did not appear. Any drop of water, bubbles and fingerprints on the bottom of the plate were removed. Then, the measurement was conducted at 450 nm using the microplate reader.

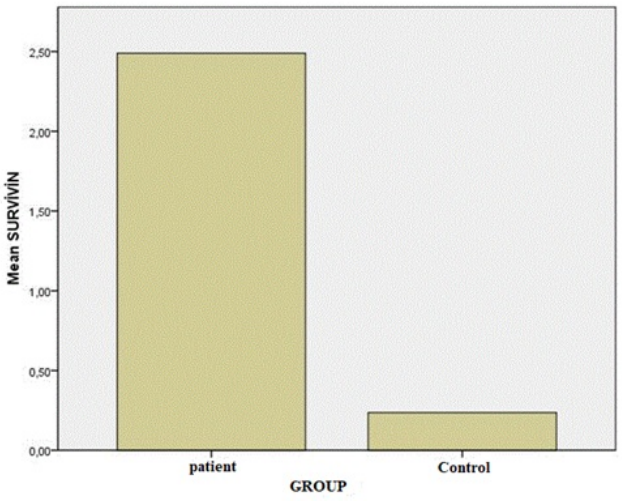

Fig. 1: Exosomal Survivia levels between patient control group

2.4. Statistical method

Statistical analyses were performed using the SPSS for Windows 15.0 software package. Visual (histograms and probability plots) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests) were used to check whether the study variables followed a normal distribution. A p value less than 0.05 on Kolmogorov-Smirnov test was considered to indicate normal distribution. Since exosomal survivin values of patient and control groups did not show a normal distribution, the groups were compared using the Mann-Whitney U test. The Kruskall-Wallis test was used to make comparisons among more than two groups in the case of non-normal distribution. Pairwise comparisons were conducted using the Mann-Whitney U test with Bonferroni correction. Overall type 1 error rate was limited using a statistical significance level of 5%.

3. Results and Discussion

A total of 75 participants including 55 patients and 20 healthy subjects were enrolled in the study. All participants were female. The mean age of the patients was 53.1±10.6 years (range, 28-76) (Table 1). A significant difference was observed between patient and control groups with respect to exosomal survivin levels (p=0.047) which were 2.48±6.38 ng/mL (range 0-40.452) for the patient group and 0.23±0.52 ng/mL (range 0-2.4) for control group (Figure 1).

A comorbid condition was present in 11 (20%) patients. The most common comorbidities were type 2 diabetes mellitus (n=2) and essential hypertension (n=3) and 3 other patients had both of these conditions. All patients had an ECOG performance status score of 0. The most common diagnostic method was tru-cut biopsy which was utilized in 39 (70%) patients. Other diagnostic methods included incisional biopsy in 6 (10.9%) patients, excisional biopsy in 9 (16.3%) patients and fine-needle aspiration biopsy in 1 (2.8%) patient. Fifty patients (90.9%) underwent surgery. Modified radical mastectomy and axillary lymph node dissection (n=39, 78%) were the most common surgical procedures. Lumpectomy was performed in 11 (22%) patients, 10 of whom underwent axillary dissection. Only one patient underwent sentinel lymph node sampling. The tumor subtypes included invasive ductal adenocarcinoma in 52 (94.5%) patients and mixed mucinous adenocarcinoma in 3 (5.5%) patients. Based on histological grading of the tumor cells, there were 3 (5.6%) Grade I patients, 17 (32.1%) Grade II patients and 33 (62.3%) Grade III patients. No significant statistical association was found between histological grade and exosomal survivin (p=0.969). Data on lymphovascular invasion (LVI) was available for 50 patients. LVI was present in 26 (52%) patients and absent in 24 (48%) patients. LVI was not statistically associated with exosomal survivin (p=0.969). Perineural invasion (PNI) data could be obtained for 50 patients. Among these evaluable patients, 21 (42%) had PNI and 29 (58%) did not. PNI was not significantly associated with exosomal survivin (p=0.806).

When the patients were evaluated for lymph node involvement, 13 (23.6%) had lymph node involvement at N1 nodes, 9 (16.4%) at N2 nodes and 6 (10.9%) at N3 nodes and 25 (45,5%) patients had none. Data on lymph node involvement were absent for 2 (3.6%) patients. There was no statistically significant association between lymph node involvement and exosomal survivin (p=0.788). Metastasis was present in 7 (12.7%) patients and absent in 48 (87.3%) patients. Of these patients, 3 had bone metastasis only, 2 had metastasis to the lungs and bones, and both liver and bone metastasis and only liver metastasis were present in one patient each. Metastasis of the tumor was not significantly

associated with exosomal survivin (p=0.242).

Based on disease stage assessment, 5 (9.1%) patients had stage I, 26 (47.3%) had stage II, 17 (30.9%) had stage III and 7 (9.1%) had stage IV cancer. No statistically significant association was observed between disease stage and exosomal survivin (p=0.175). Forty patients (72.7%) had estrogen receptor-positive and 15 (27.3%) patients had estrogen receptor-negative breast cancer. Estrogen receptor status was not significantly associated with exosomal survivin (p=0.546). 37 (72.7%) patients were progesterone receptor-positive and 18 (27.3%) were progesterone receptor-negative. Progesterone receptor status was not significantly associated with exosomal survivin (p=0.246).

When HER2 receptor status was evaluated in conjunction with fluorescent in situ hybridization (FISH) and immunohistochemical data, 18 (32.7%) patients were HER2 receptor-positive, and 37 (67.3%) patients were HER2 receptor negative. There was no significant association between HER2 receptor status and exosomal survivin (p=0.695) (Table 2). Of the patients, 26 (48.1) were premenopausal and 28 (51.9%) were postmenopausal. No statistically significant association was found between menopausal status and exosomal survivin (p=0.727). Patients were followed for a mean duration of 34.4±8.7 months (range 13.6-48.2). Median survival was not reached in survival analysis. The mean survival of the patients was 44.9±1.16 months (95% Confidence Interval (CI), 42.6-47.2) (Figure 2).

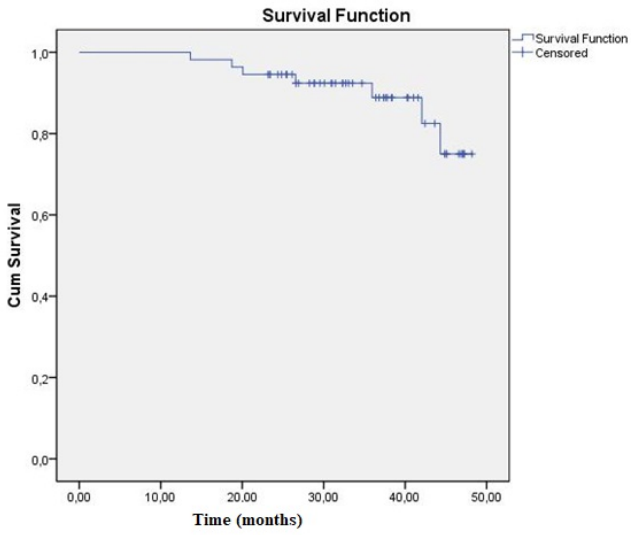

Fig. 2: Mean survival curve

4. Discussion

In the present study, breast cancer patients at different stages of the disease exhibited higher exosomal survivin levels in comparison to healthy subjects. In combination with improved breast health awareness and regular breast self-examination, periodic mammography examinations, ultrasonography, and tissue biopsy are used clinically in the early diagnosis of breast cancer. However, these modalities have several drawbacks. For example, small tumors might be missed. Thus, there is an urgent need to find a simple, accurate, precise, and non-invasive method to follow tumor activity. Shortcomings in the tools used in the early detection of breast cancer have prompted research into new diagnostic methods.[16] Extracellular Survivin has been shown to have the ability to re-enter cancer cells, increase tumor cell proliferation and apoptosis resistance.[17] Recent research on exosomes has identified several proteins associated with exosomes, including molecules required to initiate immune responses and/or apoptosis. Gunaldı et al. measured survivin levels in serum samples of breast cancer patients (accounting for 49.3% of the cancer patients studied) and healthy subjects using the ELISA assay.[18] They found that survivin values exceeding 120.8 pg/ml were associated with a 4.198-fold greater risk of cancer. Survivin was also measured using the ELISA method in the current study. However, in contrast to that study, survivin measurements were obtained from the exosomes not from the serum. This resulted in improved sensitivity as shown in Figure 1.

The role of survivin in the detection of breast cancer has also been demonstrated using research methodologies that differ from those used in our study. Wang et al. investigated the clinical value of measuring survivin and VEGF mRNA expression in the peripheral blood of breast

Table 1: General characteristics of patients

	Mean ± Standard Deviation	Median
Age (Year)	53.1±10.6	52
Blood Urea Nitrogen (mg/dl)	11.8 ±5.5	11
Creatine (mg/dl)	0.67±0.15	0.64
Aspartate Aminotransferase (U/l)	19.8±10.7	18
Alanine Aminotransferase (U/l)	20.2 ±10.2	18
Lactate Dehydrogenase (U/l)	171.8±47.8	161
Albumin (g/dl)	3.8±0.4	4
White Blood Cells (10^3/mm^3)	8.47±2.58	8.65
Neutrophil (10^3/mm^3)	5.34±2.0	5.18
Lymphocyte (10^3/mm^3)	2.35±0.87	2.4
Platelets (10^3/mm^3)	291.3±85.7	490
Hemoglobin (g/dl)	12.5±1.4	13
CA 15.3 (U/ml)	21.9±8.7	21

Table 2: Relationship between prognostic factors and exosomal survivin

	n	%	p
Grade			0,969
1	3	5,6	
2	17	32,1	
3	33	62,3	
LVI			0,969
positive	26	52	
negative	24	48	
PNI			0,806
positive	21	42	
negative	29	58	
Nodal Status			0,788
Nx	2	3,6	
N0	25	45,5	
N1	13	23,6	
N2	9	16,4	
N3	6	10,9	
Metastasis			0,242
M0	48	87,3	
M1	7	12,7	
Stage			0,175
I	5	9,1	
II	26	47,3	
III	17	30,9	
IV	7	9,1	
ER Status			0,546
positive	40	72,7	
negative	15	27,3	
PR Status			0,246
positive	37	67,3	
negative	18	32,7	
Her2 Status			0,695
positive	18	32,7	
negative	37	67,3	
Menopause			0,727
premenopausal	26	48,1	
postmenopausal	28	51,9	

(n: number, LVI: lymphovascular invasion, PNI: Perineural invasion, ER: Estrogen receptor, PR: Progesterone receptor

cancer patients by qRT-PCR.[19] They found that survivin had a sensitivity of 83.72% and 64% specificity for detection of breast cancer and VEGF had a sensitivity of 93.02% and a specificity of 88%. Moreover, they showed a positive correlation between survivin and VEGF mRNA levels. In their study, no significant association was found between survivin mRNA expression levels and clinicopathological factors which are known to have a prognostic value in breast cancer. Consistently, we did not observe a relation between exosomal survivin level and prognostic clinicopathological factors. However, other studies reported findings contradicting with aforementioned data. Xu et al. explore the relation between survivin mRNA levels obtained from formalin-fixed paraffin-embedded tumor tissues and established clinicopathological parameters.[20] High survivin mRNA expression was found to be a poor prognostic factor. Yie et al. measured survivin mRNA expression in the peripheral blood of breast cancer patients and they also identified high survivin mRNA expression as a poor prognostic factor.[21] Then, there arises a question: should survivin mRNA level be measured in blood samples or exosomes? Wang et al. suggested that whole blood survivin mRNA level might potentially serve as a biomarker for early detection of breast cancer.[19] In their study, Wang et al. used the ELISA assay to detect survivin in 15 patients and only 2 patients tested positive. They suggested that their method, quantitation of survivin mRNA, has greater sensitivity. In the current study, exosomal survivin level was non-detectable only in two patients, suggesting that our method has more sensitivity. Consistent with the relation of survivin with unfavorable clinicopathological parameters, survivin traffic within the tumor microenvironment may potentially be responsible for driving the tumor to a more aggressive phenotype, while, precluding or minimizing treatment outcomes. Thus, tumor treatment involving the use of an antibody against survivin could form immune complexes and thereby neutralize survivin extracellularly.

5. Conclusion

As a result, in our study, exosomal survivin levels were found to be statistically significantly higher in patients with breast cancer when compared to the healthy population. However, no significant relationship was found between clinicopathological parameters that are effective in breast cancer prognosis and exosomal survivin levels. The most important limitation of our study is the small number of patients. Further studies are needed to explore the characteristics of exosomal survivin.

6. Source of Funding

None.

7. Conflict of Interest

None.

References

1. Desantis CE, Ma J, Gaudet MM, Newman LA, Miller KD. Breast cancer statistics, 2019. *CA Cancer J Clin*. 2019;69(6):438–51.
2. Çiçek H, Saygılı Ö, Sever Ö, Kaya V, Ulusal H, Yıldırım M. The Diagnostic Role of A-kinase Anchoring Protein 12, Bcl-2 and High Mobility Group Box Protein-1 Levels in Breast Cancer. *J Oncol Sci*. 2019;5(3):90–5.
3. Siegel RL, Miller KD, Jemal A. Cancer statistics. *CA Cancer J Clin*. 2018;68(1):7–30.
4. Gould SJ, Raposo G. As we wait: Coping with an imperfect nomenclature for extracellular vesicles. *J Extracell Vesicles*. 2013;2. doi:10.3402/jev.v2i0.20389.
5. Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. 2014;30:255–89.

6. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med*. 1996;183(3):1161–72.
7. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. 2013;200(4):373–83.
8. Tetta C, Ghigo E, Silengo L, Deregibus MC, Camussi G. Extracellular vesicles as an emerging mechanism of cell-to-cell communication. *Endocrine*. 2013;44(1):11–9.
9. Witwer KW, Buzas EI, Bemis LT, Bora A, Lässer C, Lötvall J, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles*. 2013;2. doi:10.3402/jev.v2i0.20360.
10. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. 2014;30:255–89.
11. Oto OA, Paydas S, Tanriverdi K, Seydaoglu G, Yavuz S, Disel U. Survivin and EPR-1 expression in acute leukemias: prognostic significance and review of the literature. *Leuk Res*. 2007;31(11):1495–501.
12. Altieri DC. The case for survivin as a regulator of microtubule dynamics and cell-death decisions. *Curr Opin Cell Biol*. 2006;18:609–15.
13. Khan S, Aspe JR, Asumen MG, Almaguel F, Odumosu O, Acevedo-Martinez S, et al. cell-permeable survivin inhibits apoptosis while promoting proliferative and metastatic potential. *Br J Cancer*. 2009;100(7):1073–86.
14. Dohi T, Beltrami E, Wall NR, Plescia J, Altieri DC. Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis. *J Clin Invest*. 2004;114(8):1117–27.
15. Chugh PE, Sin SH, Ozgur S, Henry DH, Menezes P, Griffith J, et al. Systemically Circulating Viral and Tumor-Derived MicroRNAs in KSHV-Associated Malignancies. *PLoS Pathog*. 2013;9(7):e1003484. doi:10.1371/journal.ppat.1003484.
16. Umezu T, Ohyashiki K, Kuroda M, Ohyashiki JH. Leukemia cell to endothelial cell communication via exosomal miRNAs. *Oncogene*. 2013;2(22):2747–55.
17. Morton R, Sayma M, Sura MS. Economic analysis of the breast cancer screening program used by the UK NHS: should the program be maintained? . *Breast Cancer (Dove Med Press)*. 2017;9:217–25.
18. Yu X, Harris SL, Levine AJ. The regulation of exosome secretion: a novel function of the p53 protein. *Cancer Res*. 2006;66(9):4795–4801.
19. Gunaldi M, Isiksacan N, Kocoglu H, Okuturlar Y, Gunaldi O, Topcu TO, et al. The value of serum survivin level in early diagnosis of cancer. *J Cancer Res Ther*. 2018;14(3):570–3.
20. Wang S, Xu J, Zhang Q. Clinical significance of survivin and vascular endothelial growth factor mRNA detection in the peripheral whole blood of breast cancer patients. *Neoplasma*. 2016;63(1):133–40.
21. Xu C, Yamamoto-Ibusuki M, Yamamoto Y. High survivin mRNA expression is a predictor of poor prognosis in breast cancer: a comparative study at the mRNA and protein level. *Breast Cancer*. 2014;21(4):482–90.

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