Int J Clin Exp Med 2020;13(11):8609-8617
www.ijcem.com /ISSN:1940-5901/IJCEM0115724

Original Article

Clinical significance and prognostic analysis of the phosphorylation levels of Akt proteins in varying molecular subtypes of breast cancer

Lei Qiao, Chao Dong, Binlin Ma

Department of Breast and Neck Surgery, Xinjiang Medical University Affiliated Tumor Hospital, Urumqi 830011, Xinjiang, China

Received June 5, 2020; Accepted July 20, 2020; Epub November 15, 2020; Published November 30, 2020

Abstract: Objective: Use immunohistochemical approach to detect the levels of expressions of phospho-Akt (p-Akt) (Thr308) and p-Akt (Ser473) proteins in the varying molecular subtypes of breast cancer tissue, and combine clinicopathological data to analyze the relationship between Akt proteins of different activation levels and prognosis of patients with breast tumor. Methods: A total of 136 cases of tissue specimens were collected from patients with breast cancer that were hospitalized in the Breast Surgery and Head & Neck Surgery of Tumor Hospital Affiliated to Xinjiang Medical University between January 2014 and October 2014. The immunohistochemical approach was used to detect the levels of expressions of p-Akt (Thr308) and p-Akt (Ser473) proteins. The detection results were statistically analyzed with the patients' clinicopathological data and relapse-free survival (RFS). Results: The positive expression rates of p-Akt (308) and p-Akt (473) in malignant breast tumor and peritumoral tissue were 52.9% and 0% respectively, indicating that the difference was statistically significant (*P<0.05*); the expression of p-Akt (Thr308) protein was related to the tumor histological grade, expression rate of ki67, vascular tumor thrombus, size of primary tumor, molecular subtype and status of axillary lymph node of patients, indicating that the difference was statistically significant (P<0.05); the expression of p-Akt (Thr473) was related to the age, status of pausimenia, vascular tumor thrombus, size of primary tumor and status of axillary lymph node of patients, with *P<0.05*, indicating that the difference was statistically significant. The multivariate survival analysis illustrated that the 5-year RFS rate of the cases with positive expressions of both p-Akt (thr308) and p-Akt (ser473) was lower than that of patients with negative expression (*P<0.05*). Conclusion: The positive expressions of p-Akt (Thr308) and p-Akt (Ser473) are closely related to the unfavorable prognosis of patients diagnosed as breast cancer; the targeted therapy for Akt proteins is expected to be a new treatment option for patients diagnosed as breast tumor.

Keywords: Breast cancer, Akt protein, phosphorylation, prognosis

Introduction

Breast cancer is the most common malignant tumor suffered by women all over the world. The incidence increases by 3% to 4% every year in China and shows the younger trend in women [1, 2]. Based on the different expressions of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2) and Ki-67 detected by IHC approach, breast cancer is divided into four subtypes: Luminal type-A, Luminal type-B, HER-2 overexpression and triple-negative. The pathologic and clinical features and prognosis of patients with varying molecular subtyping of breast cancer are significantly different, among which the patients with Luminal breast cancer have a relatively satisfactory prognosis but are prone to lymph node metastasis, while the patients with HER-2 overexpression type and triple-negative type breast cancer are prone to distant viscera metastasis, resulting in a poor prognosis [3, 4].

Akt protein, or protein kinase B (PKB), is a serine/threonine (Ser/Thr) kinase activated by catalyzing self-phosphorylation of serine 473 (Ser473) and threonine (Thr308) [5]. As a crucial molecule in the phosphatidylinositol 3-kinase (PI3K)/Akt signal pathway, the phosphorylated Akt protein is involved in diverse physiological and pathological processes, including regulation of cell cycle and tumor angio-

genesis, and plays a role by affecting the activation state of several effector molecules downstream of the pathway [6]. This study uses immunohistochemical approach to detect the levels of protein expressions of p-Akt (Thr308) and p-Akt (Ser473) in varying molecular subtypes of breast cancer tissues, and combines clinicopathological data to analyze the relationship between Akt proteins at different activation levels and the prognosis of patients with mammary cancer.

Materials and methods

Research object

To retrospectively analyze the 136 breast cancer patients who were admitted to the Breast Surgery and Head & Neck Surgery of Tumor Hospital Affiliated to Xinjiang Medical University from January 2014 to October 2014. All the cases were females, aged 28 to 74 years, with a median age of 49.

Inclusion criteria: (1) All patients underwent radical operation or modified radical operation for breast cancer and were diagnosed as invasive ductal carcinoma by histopathology; (2) All patients were regularly re-examined in our hospital with ultrasonography, CT, MRI and bone scan. All patients with recurrence and metastasis had clear imaging and pathological diagnosis, and had complete pathological data; (3) Patients without tumor metastasis before surgery or other history of malignant tumor.

Exclusion criteria: (1) Patients with non-primary breast cancer; (2) Breast cancer patients with other malignant tumor (s); (3) Patients with other serious medical diseases; (4) Patients who received neoadjuvant chemotherapy before surgery; (5) Patients with incomplete clinicopathological and subsequent data.

Conducted the collection of clinical profile and followed-up of patients. All patients received standardized adjuvant therapy after operation in accordance with *CACA Guideline for Breast Cancer* (2019 version) [7]. Outpatient follow-up or telephone follow-up was performed on 136 patients. Among them, 106 patients had no relapse or metastasis, 30 patients had relapse or metastasis, including 8 cases of lung metastasis, 4 cases of hepatic metastatic cancer, 11 cases of bone metastatic cancer, 2 cases of chest wall recurrence, and 5 cases of metastases on axillary lymph node.

Specimen samples of para-carcinoma normal tissue next to malignant tumor from 20 cases were surgically removed within the same period as the control group. This study was carried out with the approval of the Medical Ethics Committee of the Tumor Hospital Affiliated to Xinjiang Medical University. The study obtained all informed consent of patients.

Criteria for molecular subtypes and follow-up visit

In accordance with the *CACA Guideline for Breast Cancer* (2019 version) [4], 136 patients were classified into four subtypes based on the expression of hormone receptors: 14 cases of Luminal type A (positive ER/PR, and PR≥20%, negative HER-2, Ki-67<20%); 43 cases of Luminal type B (positive ER/PR, negative HER-2, and Ki67≥20% or PR<20%; positive ER, positive HER-2, any Ki-67 or PR); 25 cases of HER-2 overexpression (negative ER and PR, positive HER-2), and 54 cases of Basal-like-type (ER, PR and HER-2 were all negative).

All the breast cancer patients were followed up regularly once every 3 months within the 2 years of treatment, once every 6 months for 3-5 years after treatment, and once-a-year for more than 5 years after surgery until death or suspension of the follow-up. All follow-up work on patients was ended as of October 2019.

Experimental methods and procedures

The expressions of p-Akt (Thr308) and p-Akt (Ser473) in breast carcinoma tissue and breast gland tissue was detected by immunohistochemistry. The tissue sections of breast cancer and breast gland were placed in the oven at 65°C for 2 hours, dewaxed with xylene for 40 min, and then dehydrated with gradient ethanol solution (80% to 90% - anhydrous). After rinsing the tissue sections with water, 3% hydrogen peroxide was added to remove endogenous peroxidase. Added EDTA buffer solution (pH8.0) to repair the antigen at 95°C for 15 minutes, and then cooled and rinsed at room temperature. After rinsing for 3 times, 3 min/time with PBS solution, tissue sections were sealed with 5% FBS at normal temperature for 20 min. The rabbit anti-human p-Akt (308) polyclonal anti-

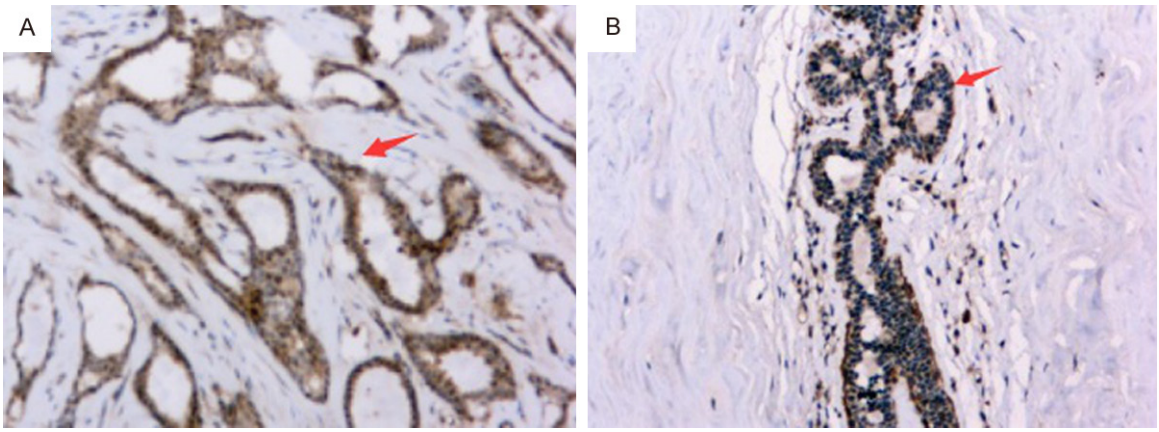

Figure 1. Expression of p-Akt (308) in malignant breast tumor and peritumoral tissue. The positive expression rates of p-Akt (308) in malignant breast tumor and para-carcinoma normal tissue were 52.9% and 0% respectively, for which the difference was statistically significant with *P<0.05.*

body diluted at 1:200 (sc-16646-R, bought from Santa Cruz Biotechnology Co., Ltd.) and rabbit anti-human p-Akt (ser473) monoclonal antibody diluted at 1:50 (#4060, bought from Cell Signal Technology Co., Ltd.) were added and placed overnight at 4°C and then rinsed 3 times with PBSby 3 min/time. The PowerVision two-step method was used to drip horseradish peroxidase-labeled poly anti-rabbit IgG (purchased from Leica Microsystems) at room temperature for 30 min. Rinse 3 times with PBSby, 3 min/time, and followed by rinse with running water after 10 minutes of DAB color development. The sections were counterstained with hematoxylin, dehydrated with ethanol, transparent with xylene, and then sealed. The breast cancer tissues with known positive expression were taken as the positive control (the source was the filed wax blocks of pathology from the hospital where the experiment was conducted).

Judgement of results

The protein expressions of p-Akt (Thr308) and p-Akt (Ser473) were located in the nucleus or cytoplasm, with brownish yellow or brown particles as positive staining. The histological score method (H-score) was used to interpret the results. Scores of percentage of positive cells (pp): 10 hpf (×400) were stochastically selected under an optical microscope. One hundred tumor cells were counted in each field to calculate the PP. <10% for 0 point, 10% to 25% for 1 point, 26% to 50% for 2 points, 51% to 75% for 3 points, >75% for 4 points. Scores of tinting strength: 0 point represented for negative staining, 1 point represented for poor staining, 2 points for medium-level tinting, and 3 points for high tinting strength. H-score = PP × SI, 0 to 4 points was negative expression and 5 to 12 points was positive expression. The judgment of results to all specimens was confirmed by two senior physicians in the pathology department.

Statistical method

The experiment data were analyzed using the latest version 20.0 of SPSS statistical software. The specific methods adopted were as follows: X^2 test or Fisher exact test for enumeration data, rank-sum test for grading data, Kaplan-Meier method for drawing of the survival curve, log-rank test for component contrast of survival analysis, and the COX regression model for analyzing the independent prognostic factor. *P<0.05* indicated that the difference has statistical significance.

Results

Expressions of p-Akt (Thr308) and p-Akt (Ser473) proteins in malignant breast tumor and para-carcinoma normal tissue

The positive expression rates of p-Akt (308) in malignant breast tumor and para-carcinoma normal tissue were 52.9% and 0% respectively, for which the difference had statistical significance with *P<0.05,* see **Figure 1**. The positive expression rates of p-Akt (473) in malignant

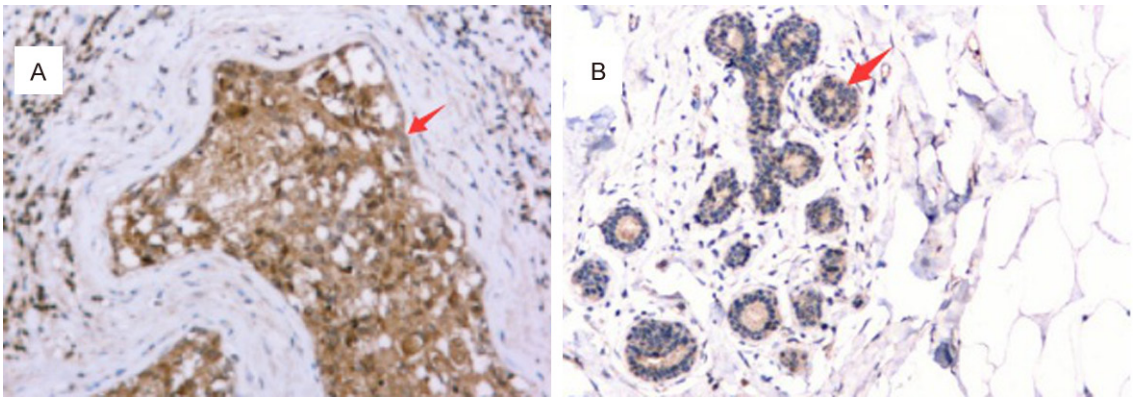

Figure 2. Expression of p-Akt (473) in malignant breast tumor and peritumoral tissue. The positive expression rates of p-Akt (473) in malignant breast tumor and para-carcinoma normal tissue were 62.5% and 0% respectively, for which the difference was statistically significant with *P<0.05.*

Table 1. The relationships between the expressions of p-Akt (Thr308) and p-Akt (Ser473) proteins and clinicopathologic characteristics of breast cancer patients

Clinicopathologic characteristics	p-AKT (Thr308)		X^2	*P*	p-AKT (Ser473)		X^2	*P*
	Positive	Negative			Positive	Negative		
Age								
≤50	44	34	0.883	0.347	56	22	6.742	0.009
>50	28	30			29	29		
Status of pausimenia								
Yes	21	26	1.967	0.161	24	23	4.008	0.045
No	51	38			61	28		
Tumor histology								
I	1	6	13.085	0.000	4	3	2.458	0.110
II	33	42			43	32		
III	38	16			38	16		
Expression rate of ki67								
<20%	1	13	13.139	0.000	6	8	2.569	0.109
≥20%	71	51			79	43		
Vascular tumor thrombus								
Yes	62	8	73.501	0.000	51	19	6.602	0.010
No	10	56			34	32		
Size of primary tumor								
≤2 cm	16	56	57.952	0.000	37	35	8.059	0.005
>2 cm	56	8			48	16		
Molecular subtype								
Luminal type A	1	13	48.021	0.000	6	8	4.924	0.058
Luminal type B	12	31			25	18		
HER-2 positive	13	12			15	10		
Triple-negative	46	8			39	15		
Status of axillary lymph node								
Negative	6	56	85.608	0.000	32	30	5.763	0.016
Positive	66	8			53	21		

breast tumor and para-carcinoma normal tissue were 62.5% and 0% respectively, for which the difference had statistical significance with *P<0.05*, see **Figure 2**.

Table 2. Univariate survival analysis of factors influencing relapse-free survival (RFS) of breast cancer patients (N=136)

Clinicopathologic characteristics	5-year RFS rate (%)	*P*
Age		
≤50	78.2	0.934
>50	77.6	
Pausimenia		
Yes	80.9	0.482
No	76.4	
Histology		
I	75	0.228
II	85.1	
III	68.5	
ki67		
<20%	92.9	0.280
≥20%	76.2	
Vascular tumor thrombus		
Yes	58.6	0.000
No	98.5	
Tumor size		
≤2 cm	98.6	0.000
>2 cm	56.3	
Molecular subtype		
Luminal type A	92.9	0.000
Luminal type B	88.4	
HER-2 positive	72	
Triple-negative	68.5	
p-AKT (Thr308)		
Negative	98.4 72	0.000
Positive	59.7 64	
p-AKT (Ser473)		
Negative	96.1	0.000
Positive	67.1	
p-AKT (Thr 308) & (Ser473)		
-/-	100	0.000
-/+	95.3	
+/+	41.3	

The relationship between the expressions of p-Akt (Thr308) and p-Akt (Ser473) proteins and the clinical and pathologic characteristics of breast cancer patients

The protein expression of p-Akt (Thr308) in breast cancer tissue was related to the tumor histological grade, expression rate of ki67, vascular tumor thrombus, size of primary tumor, molecular subtype and status of axillary lymph node of patients, *for which* the difference has statistical significance with P<0.05; while it was unrelated to the age and status of pausimenia of patients with *P>0.05,* indicating that the difference has no statistical significance. The expression of p-Akt (Thr473) in breast cancer tissue was related to the age, status of pausimenia, vascular tumor thrombus, morphology of primary tumor, and status of axillary lymph node of patients with *P<0.05,* indicating a statistically significant difference; while it was unrelated to the histologic grading, expression rate of ki67 and molecular subtype, with *P> 0.05,* indicating that there was no statistically significance of difference, see **Table 1**.

Univariate analysis of prognosis to breast cancer disease

The median follow-up period of 136 patients with breast cancer was 54.61 months (14 to 60 months) in the study and no cases was lost of follow-up. Among which, there were 106 patients without recurrent metastasis and 30 patients with recurrent metastasis, including 8 patients with pulmonary metastasis, 4 patients with hepatic metastatic cancer, 11 patients with bone metastatic cancer, 2 patients with chest wall recurrence, and 5 patients with axillary lymph node metastasis. The RFS rates of 1-year, 3-year and 5-year were 99.2%, 86.7% and 78% respectively.

The results of univariate survival analysis indicated that the relapse-free survival of patients with breast tumor was related to the primary tumor size, molecular subtypes, vascular tumor thrombus, status of axillary lymph node and tumor histological grade. *P<0.05, which* indicated that the difference was statistically significant. See details elaborated in **Tables 2**, **3**.

The analysis of Kaplan-Meier survival curve (**Figure 3**) indicated that the 5-year RFS rates of the group with positive expression of p-AKT (Thr308) protein and negative expression of p-AKT (Thr308) protein were 59.7% and 98.4% respectively, with the difference has statistical significance (*P<0.05)*; the 5-year RFS rates of the group with positive and negative expression of p-AKT (Thr473) protein were 67.1% and 96.1% respectively, with the difference has statistical significance *(P<0.05).* The group with both negative expression of p-AKT (Thr308) and p-AKT (Thr473) proteins and positive ex-

Table 3. Multivariate survival analysis (COX proportional hazard model) of factors influencing relapse-free survival (RFS) of breast cancer patients

Clinicopathological Features	Hazard Ratio	95% *CI*	*P* value
Vascular tumor thrombus	12.15	4.72-25.90	
Tumor size	16.76	4.78-84.34	
Molecular subtype		4.15-23.83	0.002
Status of axillary lymph node	11.28		0.007
Luminal type A	7.25		0.028
Luminal type B	0.21	0.192-14.06	0.001
HER-2 positive	1.99	0.555-36.69	
Triple-negative	2.6	0.7-39.55	
p-Akt (Thr308)	11.72	4.44-23.37	0.001
p-Akt (Ser473)	9.859	2.37-41.89	0.002
p-Akt (Thr308) & (Ser473)			0.004
-/-	22.8		
-/+	2.43	4.36-58	
+/+	6.72	7.99-59	

pression were marked as the "-/-" group and "+/+" group respectively. The group with expression of any protein was recorded as "-/+" group. According to the further analysis, the 5-year RFS rates of "-/-" group, "-/+" group, and "+/+" group were 100%, 95.3%, and 41.3 respectively. The difference had statistical significance with *P<0.05*.

Multivariate analysis to prognosis of breast cancer patients

The factors that were statistically significant as shown in the univariate survival analysis were included into the COX regression model. The computation showed that the size of primary tumor, molecular subtype, vascular tumor thrombus, status of axillary lymph node, p-Akt (Thr308) and p-Akt (Ser473) proteins were all independent hazard factors that affect the RFS rate of breast cancer patients after surgery (**Table 3**).

Discussion

As a systemic disease, breast malignant tumor has been increasingly treated with systematic medical techniques at present. However the patients are still burdened with the risk of long-term recurrence and metastasis. Among the varying molecular subtypes of breast cancers, luminal type B and HER-2 positive breast cancers are prone to lymph node metastasis [8], while breast cancer with triple-negative is the subtype with the worst prognosis as endocrine therapy and targeted therapy for HER-2 molecules have no effect on it and is prone to distant organ metastasis [9]. Therefore, it is a hot topic to search for effective prognosis predictors and potential therapeutic targets for breast cancer.

The abnormal activation of PI3K/Akt signaling pathway has close connection with the formation of tumor and the acceleration of tumor metastasis [10, 11]. As a downstream substrate of phosphatidylinositol 3-kinase (PI3K), Akt can be transmitted through signaling pathways, and the various growth factors can accurately play their role and function. Phosphorylation is the main method of Akt activation. The activated PI3K can catalyze the phosphorylation of phosphatidylinositol to produce the second form of phosphatidylinositol-3,4,5-triphosphate (PIP3) [12]. PIP3 can bind to the PH region of Akt, and the latter will undergo translocation after the binding. Akt will transfer from cytoplasm to cell membrane and promote its conformational change [13]. In fact, the activation of AKT requires two Ser/Thr protein kinases. One is 3-phosphate inositol-dependent protein kinase 1 (PDK1), which translocates to the cell membrane with its PH domain to phosphorylate threonine (Thr308) loci on the Akt kinase. The other is serine 473 (Ser473) on the p-Akt kinase of PDK2 (usually mTOR). The Akt proteins can be fully activated after the above loci are both phosphorylated. When it is fully phosphorylated, Akt protein kinase can enter into cell matrix or nucleus, and regulate gene transcription, translation, material and energy metabolism, cell proliferation and apoptosis, and other biological effect through phosphorylation of its downstream substrate molecules [14, 15]. Therefore, the phosphorylation of both Thr308 and Ser473 loci of Akt protein kinase is considered as a sign of the activation of Akt proteins.

In this study, the immunohistochemical approach was used to detect the levels of expressions of activated Akt proteins, p-Akt (Thr308) and p-Akt (Ser473) proteins in the varied molecular subtypes of breast cancer tissue, and

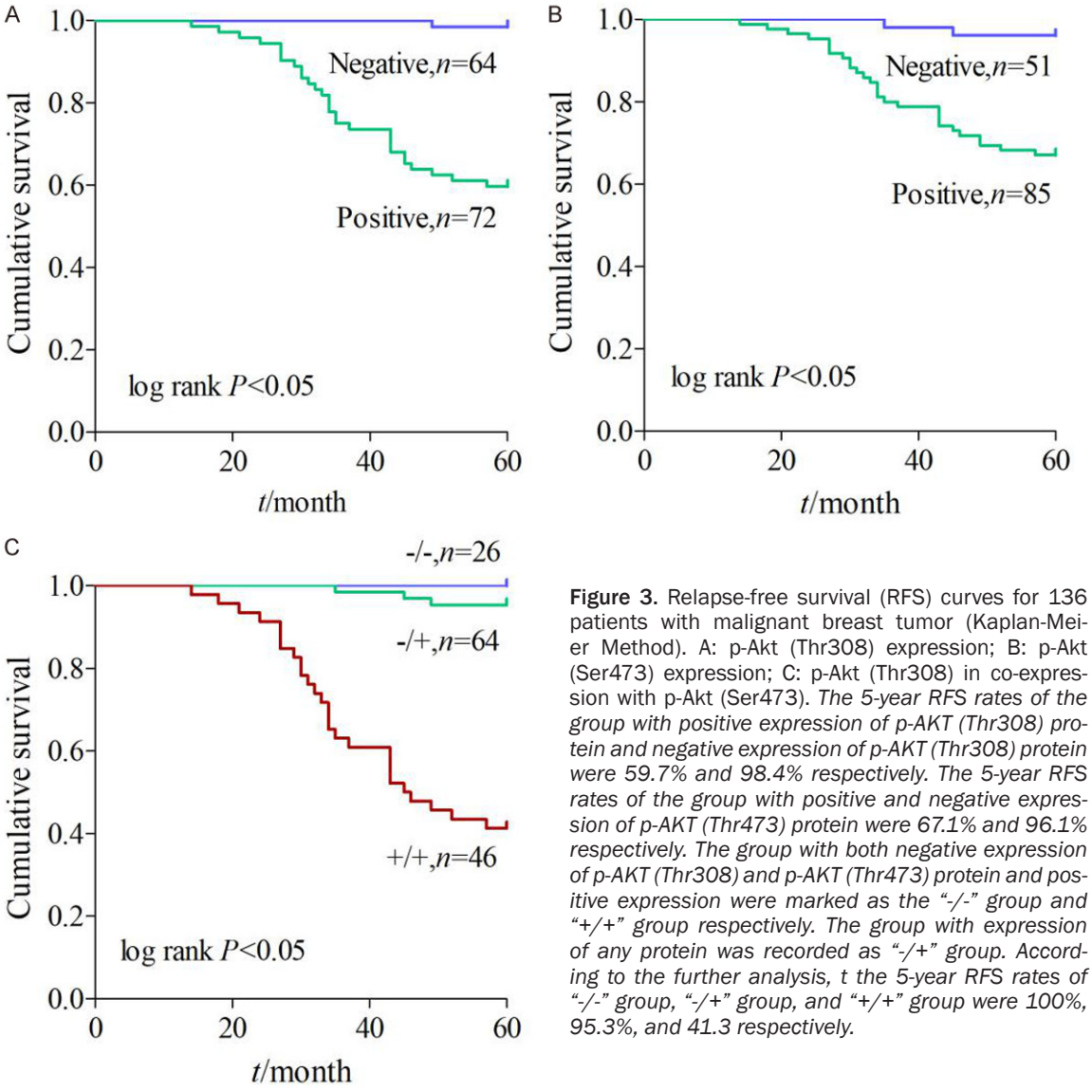

Figure 3. Relapse-free survival (RFS) curves for 136 patients with malignant breast tumor (Kaplan-Meier Method). A: p-Akt (Thr308) expression; B: p-Akt (Ser473) expression; C: p-Akt (Thr308) in co-expression with p-Akt (Ser473). *The 5-year RFS rates of the group with positive expression of p-AKT (Thr308) protein and negative expression of p-AKT (Thr308) protein were 59.7% and 98.4% respectively. The 5-year RFS rates of the group with positive and negative expression of p-AKT (Thr473) protein were 67.1% and 96.1% respectively. The group with both negative expression of p-AKT (Thr308) and p-AKT (Thr473) protein and positive expression were marked as the "-/-" group and "+/+" group respectively. The group with expression of any protein was recorded as "-/+" group. According to the further analysis, t the 5-year RFS rates of "-/-" group, "-/+" group, and "+/+" group were 100%, 95.3%, and 41.3 respectively.*

analyzed its relationship with clinicopathologic characteristics of patients. The results showed that the expression levels of Akt (p-Akt) (Thr308) and p-Akt (Ser473) proteins in breast cancer tissues were higher than those in tissues of normal breast gland; the protein expression of p-Akt (Thr308) was related to the tumor histological grade, expression rate of ki67, vascular tumor thrombus, size of primary tumor, molecular subtype and status of axillary lymph node of patients; and the protein expression of p-Akt (Thr473) was related to the age, pausimenia status, vascular tumor thrombus, the dimensions of primary tumor and status of axillary lymph node; further analysis revealed that the positive expression rate of p-Akt (Thr308) and p-Akt (Ser473) proteins in triple-negative breast cancer tissue was significantly higher than that in non-triple-negative breast cancer. Similar to the research reports of other scholars [16, 17], the p-Akt level in triple negative breast cancer was significantly higher than that in other subtypes, suggesting the apparent activation of Akt in triple-negative breast cancer. This pointed out that the fully activated Akt protein has a great effect on the occurrence and development of breast cancer.

To clarify the connection for the phosphorylation levels of Akt proteins and prognosis of breast cancer to patients, the univariate survival analysis of this study showed that single

positive expression of Akt at Thr308 and Ser473 loci or the positive expression of both of them were both the poor prognostic factors for patients with breast cancer. Furthermore, it adopted COX regression model to analyze and found that the size of primary tumor, molecular subtype, vascular tumor thrombus, status of axillary lymph node, and the positive expressions of p-Akt (Thr308) and p-Akt (Ser473) proteins were all independent hazard factors of postoperative RFS rate for patients with breast cancer. It has been shown ion previous studies that the abnormal phosphorylation of Akt in breast cancer tissue was often associated with the deficiency of phosphatase and tensin homologuedeleted on chromosome ten (PTEN) [18]. As a tumor suppressor gene, PTEN gene has phosphatase activity. Its lipid phosphatase activity can generate phosphatidylinositol diphosphate (PIP2) by dephosphorylating the lipid substrate phosphatidylinositol triphosphate (PIP3) at the D3 position, so as to reverse the phosphorylation effect of PI3K on Akt, negatively regulate PI3K/Akt signal, inhibit the proliferation of tumor cells, and induce their apoptosis [19]. Furthermore, Akt protein kinases in phosphorylated state can directly phosphorylate Thr145 of p21 protein (a kind of cyclin-dependent kinase (CKDs) inhibitor); while the nuclear localization signal (NLS) of Thr145 that phosphorylated near p21 protein can prevent p21 from binding to nuclear import protein and retain p21 in the cytoplasm; besides, phosphorylated Akt can also indirectly phosphorylate Ser21 of p21 by activating protein kinase C (PKC), thereby inhibiting p21 in the cytoplasm and prolonging the half-life period life of p21 [20]. The phosphorylation of Thr145 and Ser-146 of P21 protein can prevent p21 from binding to Proliferating Cell Nuclear Antigen (PCNA) in the nucleus, and the free PCNA will activate DNA polymerase and accelerate S phase operation [21]. This is likely the mechanism that phosphorylated Akt proteins regulate p21 in the cytoplasm to promote the malignant proliferation of tumor cells.

The research data may be biased due to the small sample size in this study; besides, as its specific mechanism is not analyzed, it is necessary to further study it in the future.

In conclusion, by combining with Chinese and foreign literature reports and the results of this study, the author holds the opinion that the deficiency of PTEN genes in the breast cancer tissue and the action of related factors in the tumor microenvironment cause the abnormal phosphorylation of Akt proteins. When both Thr308 and Ser473 are phosphorylated, the Akt proteins will be fully activated to regulate multiplication and attack breast cancer cells by phosphorylating downstream p21 proteins. Therefore, the positive expression of p-Akt (Thr308) and p-Akt (Ser473) can be used as a predictor for the poor prognosis of breast cancer patients, and the targeted therapy for Akt is expected to be a new treatment option for patients with triple-negative breast cancer.

Acknowledgements

Xinjiang Medical University Research and Innovation Fund Project (No: XYDCX201680).

Disclosure of conflict of interest

None.

Address correspondence to: Binlin Ma, Department of Breast and Neck Surgery, Xinjiang Medical University Affiliated Tumor Hospital, No. 789 East Suzhou Street, Xinshi District, Urumqi 830011, Xinjiang, China. Tel: +86-0991-7819072; E-mail: mabinlin21@163.com

References

[1] Saei Ghare Naz M, Simbar M, Rashidi Fakari F and Ghasemi V. Effects of model-based interventions on breast cancer screening behavior of women: a systematic review. Asian Pac J Cancer Prev 2018; 19: 2031-2041.

[2] Zubor P, Kubatka P, Kajo K, Dankova Z, Polacek H, Bielik T, Kudela E, Samec M, Liskova A, Vlcakova D, Kulkovska T, Stastny I, Holubekova V, Bujnak J, Laucekova Z, Büsselberg D, Adamek M, Kuhn W, Danko J and Golubnitschaja O. Why the gold standard approach by mammography demands extension by multiomics? Application of liquid biopsy mirna profiles to breast cancer disease management. Int J Mol Sci 2019; 20: 2878.

[3] Brozovich FV, Nicholson CJ, Degen CV, Gao YZ, Aggarwal M and Morgan KG. Mechanisms of vascular smooth muscle contraction and the basis for pharmacologic treatment of smooth muscle disorders. Pharmacol Rev 2016; 68: 476-532.

[4] Yan W, Ma X, Zhao X and Zhang S. Baicalein induces apoptosis and autophagy of breast

cancer cells via inhibiting PI3K/AKT pathway in vivo and vitro. Drug Des Devel Ther 2018; 12: 3961-3972.
[5] Du L, Li X, Zhen L, Chen W, Mu L, Zhang Y and Song A. Everolimus inhibits breast cancer cell growth through PI3K/AKT/mTOR signaling pathway. Mol Med Rep 2018; 17: 7163-7169.
[6] McKenna M, McGarrigle S and Pidgeon GP. The next generation of PI3K-Akt-mTOR pathway inhibitors in breast cancer cohorts. Biochim Biophys Acta Rev Cancer 2018; 1870: 185-197.
[7] Chinese Anti-Cancer Association, Committee of Breast Cancer Society (CACA-CBCS). CACA Guideline for Breast Cancer (2019 version). China Oncology 2019; 12: 609-680.
[8] Chung MJ, Lee JH, Kim SH, Suh YJ and Choi HJ. Simple prediction model of axillary lymph node positivity after analyzing molecular and clinical factors in early breast cancer. Medicine (Baltimore) 2016; 95: e3689.
[9] Wang R, Li X, Zhang H, Wang K and He J. Cell-free circulating tumor DNA analysis for breast cancer and its clinical utilization as a biomarker. Oncotarget 2017; 8: 75742-75755.
[10] Lu Y, Bradley JS, McCoski SR, Gonzalez JM, Ealy AD and Johnson SE. Reduced skeletal muscle fiber size following caloric restriction is associated with calpain-mediated proteolysis and attenuation of IGF-1 signaling. Am J Physiol Regul Integr Comp Physiol 2017; 312: R806-R815.
[11] Gao W, Wu XL, Li DZ and Liu HD. HOTTIP participates in mammary cancer by promoting cell proliferation via PI3K/AKT pathway. Eur Rev Med Pharmacol Sci 2018; 22: 4181-4187.
[12] Sharma VR, Gupta GK, Sharma AK, Batra N, Sharma DK, Joshi A and Sharma AK. PI3K/Akt/mTOR intracellular pathway and breast cancer: factors, mechanism and regulation. Curr Pharm Des 2017; 23: 1633-1638.
[13] He LF, Xu HW, Chen M, Xian ZR, Wen XF, Chen MN, Du CW, Huang WH, Wu JD and Zhang GJ. Activated-PAK4 predicts worse prognosis in breast cancer and promotes tumorigenesis through activation of PI3K/AKT signaling. Oncotarget 2017; 8: 17573-17585.
[14] Hatem R, El Botty R, Chateau-Joubert S, Servely JL, Labiod D, de Plater L, Assayag F, Coussy F, Callens C, Vacher S, Reyal F, Cosulich S, Diéras V, Bièche I and Marangoni E. Targeting mTOR pathway inhibits tumor growth in different molecular subtypes of triple-negative breast cancers. Oncotarget 2016; 7: 48206-48219.
[15] Mathe A, Scott RJ and Avery-Kiejda KA. miRNAs and other epigenetic changes as biomarkers in triple negative breast cancer. Int J Mol Sci 2015; 16: 28347-28376.
[16] Li N, Kong J, Lin Z, Yang Y, Jin T, Xu M, Sun J and Chen L. Ezrin promotes breast cancer progression by modulating AKT signals. Br J Cancer 2019; 120: 703-713.
[17] Jiang H, Zhang X, Tao Y, Shan L, Jiang Q, Yu Y, Cai F and Ma L. Prognostic and clinicopathologic significance of SIRT1 expression in hepatocellular carcinoma. Oncotarget 2017; 8: 52357-52365.
[18] Héron-Milhavet L, Franckhauser C, Rana V, Berthenet C, Fisher D, Hemmings BA, Fernandez A and Lamb NJ. Only Akt1 is required for proliferation, while Akt2 promotes cell cycle exit through p21 binding. Mol Cell Biol 2006; 26: 8267-8280.
[19] Iwahori S, Murata T, Kudoh A, Sato Y, Nakayama S, Isomura H, Kanda T and Tsurumi T. Phosphorylation of $p27^{Kip1}$ by epstein-barr virus protein kinase induces its degradation through SCF^{Skp2} ubiquitin ligase actions during viral lytic replication. J Biol Chem 2009; 284: 18923-18931.
[20] Jia L, Huang S, Yin X, Zan Y, Guo Y and Han L. Quercetin suppresses the mobility of breast cancer by suppressing glycolysis through Akt-mTOR pathway mediated autophagy induction. Life Sci 2018; 208: 123-130.
[21] Xie F, Jin K, Shao L, Fan Y, Tu Y, Li Y, Yang B, van Dam H, Ten Dijke P, Weng H, Dooley S, Wang S, Jia J, Jin J, Zhou F and Zhang L. FAF1 phosphorylation by AKT accumulates TGF-β type II receptor and drives breast cancer metastasis. Nat Commun 2017; 8: 15021.