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Study on correlation between PKIB and pAkt expression in breast cancer tissues

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Abstract. – OBJECTIVE: This study is to explore the expression of cyclic AMP (cAMP) dependent protein kinase inhibitor (PKIB) in human breast cancer and the correlation with phosphorylated protein kinase B (pAkt) expression in the tumor tissues.

MATERIALS AND METHODS: Surgical removal of the tissue samples from 148 patients with primary breast cancer from 2011-2015 were selected, and then we detected the PKIB, estrogen receptor (ER), progesterone receptor (PR) and proto oncogene (HER2) by using immunohistochemical technique and the Allred score classification standard. The clinical pathological factors such as tumor diameter, lymph node metastasis and tumor stage, etc. were analyzed statistically. Then we detected that the PKIB and pAkt respectively of immunohistochemical expression and cellular localization of four subtypes in patients which were luminal A, luminal B, HER2+/ER-type and triple negative breast cancer type.

RESULTS: Immunohistochemistry staining showed when pAkt was positive and there were significant correlations with the expression of PKIB ($p<0.05$). Both positive staining reactions occurred in the cytoplasm of the tumor. Histopathological type, tumor diameter, lymph node metastasis, tumor stage and other clinical pathological factors were not significantly associated with the expression of PKIB. In addition, the expression of PKIB was also significantly associated with the triple negative breast cancer in the four subtypes ($p<0.05$).

CONCLUSIONS: The expression of PKIB in the cytoplasm of tumor is closely related to pAkt and the triple negative breast cancer. It was concluded that the PKIB promoted the occurrence and development of breast tumors by regulating the Akt signaling pathway. PKIB will be a potential therapeutic target for breast cancer, especially in the diagnosis and treatment of triple negative breast cancer.

Key Words
Breast cancer, PKIB, pAkt, Triple negative breast cancer, Immunohistochemistry.

Introduction

In the western developed countries, the occurrence rate of breast cancer is more than 60/10 million, which makes breast cancer the most common malignant tumor in women[1]. At present, the average annual incidence rate of breast cancer in China is about 30/10 million, and there is an increasing trend year by year[2]. DNA microarray gene expression studies confirmed that a variety of breast cancer subtypes have significant differences in the prognosis and therapeutic targets[3]. The breast cancer is divided into two types according to the expression of the hormone receptor dependent genes. One type is estrogen receptor (ER) and progesterone receptor (PR) double negative breast cancer (DNBC), including the basal cell-like subtype of human epidermal growth factor receptor-2 (HER2) with or without the expression of human epidermal growth factor receptor and HER2 positive expression subtype. Another breast cancer is composed of ER and PR positive expression of tumor; luminal A (HER2 negative) and luminal B (HER2 positive) subtypes were also included[4]. The latest study found that ER, PR, and ER2 are common in young African American women with a negative prognosis of triple negative breast tumors[5]. However, for the estrogen sensitive luminal type A tumor, the prognosis of patients is generally more ideal[6]. Serine/threonine protein kinase Akt can promote the cell growth and proliferation, inhibit the process of promoting apoptosis and stimulate angiogenesis after activation or phosphorylation[7]. In a variety of tumor tissues, the Akt pathway is considered to be the key pathway for the regulation of tumor invasion and metastasis, and it has become a promising therapeutic target in the treatment of cancer[8]. The study confirmed that the expression level of phosphorylated Akt (pAkt) kinase was

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significantly increased in triple negative breast cancer (TNBC). TNBC includes basaloid type, apocrine carcinoma, metaplastic carcinoma and myoepithelial differentiation cancer and a variety of different types of cancer[9]. The activation of Akt kinase may lead to tumor proliferation and result in poor prognosis in patients with breast cancer. In addition, as a hormone-dependent tumor, the growth of breast cancer cells is regulated by a variety of hormones. In the process of occurrence and development of the breast cancer, the estrogen plays an important role, and the endocrine therapy can inhibit the growth of tumor cells by decreasing the level of estrogen in the body or inhibiting the action of estrogen. However, the Akt activation can significantly inhibit the effect of endocrine therapy, and pAkt in the endocrine therapy for breast cancer resistance is a very valuable potential predictive factor. Cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) is generally considered to be a key factor in the regulation of physiological or pathological changes in the body mediated by cAMP[10]. When combined with the protein G, PKA activates a variety of ligand receptor signaling pathways that control the cell growth and the differentiation. In patients with breast cancer, the presence of endocrine therapy resistance is highly correlated with the activation of the PKA pathway. In addition, clinical researches also found that the resistance of anti estrogen drugs tamoxifen taken by breast cancer patients was correlated with the activation of PKA[11,12]. In addition, the activation of PKA signaling pathway in HER2 positive breast cancer is believed to be the basis of drug resistance to chemotherapy[13].

PKA has three inhibitory factors: PKI-α, PKI-β, and PKI-γ. These factors express and regulate the PKA pathway in breast tumors[14,15]. PKI-β, also known as PKIB, its inhibitory effect on PKA is still unclear. Nevertheless, recent studies found that PKIB may be the core regulator of PKA pathway[16]. PKIB was overexpressed in castration-resistant prostate cancer and highly correlated with Gleason score[17]. Phosphorylation of Akt is induced by functional connectivity between PKA and Akt pathways, thus highlighting PKIB as a predictor of malignant phenotype and poor prognosis in prostate cancer. This study mainly discussed the correlation between the expression of PKIB and pAkt in the breast cancer, especially the TNBC subtypes, to provide an objective index for further clinical research.

Patients and Methods

Breast Cancer Tumor Tissue Sample Collection

The samples of 148 patients with primary breast cancer who underwent surgical treatment at The Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, Henan, China) were collected for 2011-2015. The average age of the patients was 61 years old (range 33-90) by inquiring the clinical data of the patients in the hospital. The breast tumor tissue samples were fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin eosin (H&E) staining. All the specimens were 5 μm serial sections.

Immunohistochemical Method

The EnVision two-step method was used in immunohistochemistry. 5 μm sections were dewaxed to water and treated with 0.3% hydrogen peroxide methanol for 10 minutes after washing. The section were then put in microwave oven for antigen repair for 20 min with the rabbit anti-human PKIB (1:80 dilution), pAkt (1:80 dilution) polyclonal antibody, mouse anti-human HER2 (1:50 dilution), ER (1:50 dilution) and PR (1:50 dilution) monoclonal antibody added in succession. All materials were purchased from Abcam (Cambridge, MA, USA). The first antibody was placed at 4°C overnight and, then, it was washed 3 times with phosphate buffered saline (PBS), 2 minutes for each. The two-step method was used by DAB color and rabbit/mouse general type immunohistochemistry ELISA Kit (Beijing Jiuzhou Tianrui Technology Co., Ltd., Beijing, China). The sections were then washed by tap water, counterstained with hematoxylin for 10 min (70%-100%) levels of alcohol dehydration, 3 minutes for each level. After that, the sections were placed in xylene for 5 minutes, finally mounted and observed under microscope. PBS buffer solution was used as negative control instead of the first antibody, and the breast cancer positive samples (Beijing Zhongshan Jinqiao Inc. Beijing, China) were used as positive control. Each tumor sample was independently tested for three times to verify the reliability of the results. CX31-LV320 OLYMPUS microscope was purchased from Beijing Changhen Rongchuang Technology Co., Ltd. (Beijing, China).

Standard and Subtype Definition of Immunohistochemistry

In order to detect the expression of PKIB, the Allred score method was used to determine the

staining intensity of each sample in our hospital. According to the immune staining intensity of pAkt antibody in cytoplasm, ER in nucleus, the PR antibody and the records were marked as negative (-) or positive (+). The expression of HER2 was assessed according to the intensity and integrity of its membrane staining and recorded as negative (-) or positive (+). In addition, for a variety of subtypes of tumor, the formalin fixed and paraffin embedded tissue samples by using gene expression analysis was not ideal; therefore, this study used immunohistochemistry to detect the difference between subtypes of breast cancer. According to the immunohistochemistry, the breast cancer subtypes can be defined as follows: luminal A type (ER+ and/or PR+, HER2-) and luminal B type (ER+ and/or PR+, HER2+), HER2+/ER-type (ER-, PR-, and HER2+) and TNBC (ER-, PR, and HER2-).

Statistical Analysis

We used SPSS17.0 (SPSS Inc., Chicago, IL, USA) for data analysis. The χ^2-test was used to analyze the relationship between the expression of PKIB and pAkt, ER, PR and HER2, and its correlation with the clinical features of the patients. $p<0.05$ difference has statistical significance.

Results

Positive Expression of pAkt Was Significantly Associated with PKIB

By analyzing the clinical pathological data of 148 cases of primary breast cancer, we found that when pAkt positive expressed and PKIB expression was significantly associated ($p<0.05$), but other factors such as histological type, tumor diameter, lymph node metastasis, tumor stage and other factors were not significant, as shown in Figure 1.

Overexpression of PKIB in the Cytoplasm of Breast Tumors and Its Association with pAkt

Immunohistochemical staining found that there was a strong immune response of PKIB antibodies in cytoplasm in 53.1% (34/64 cases) of breast cancer patients, the staining of which was found to be significant. In contrast, strong cytoplasmic staining was visible in the immune response of 46.9% (30/64, Figure 1A) of the tumor cells. In normal breast tissues, PKIB immunoreactivity was limited to the minimal extent of breast cells. The rate of positive staining of anti pAkt antibody in tumor was 18.2% (27 cases, Figure 1B), including 20 tissue samples with PKIB positive expression (Figure 1C). In spite of the relatively low expression of pAkt, it was found that there was a significant relationship correlation PKIB and pAkt immunoactivities ($p<0.05$).

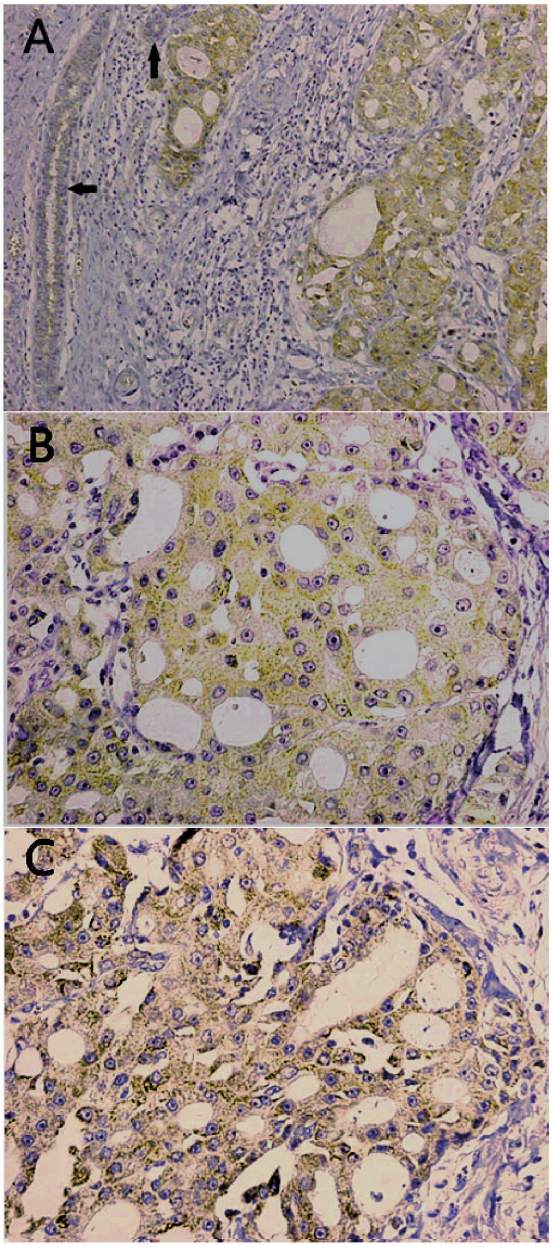

Figure 1. Expression of PKIB and pAkt in breast tumor cytoplasm. **A**, In breast tumor tissues, the anti PKIB antibody has a uniform and strong immunoactivity and only an extremely weak cytoplasmic immunoactivity occurs in normal ductal epithelial cells *(arrow pointing)*, x150. **B**, Anti pAkt antibody in breast tumor cytoplasm showed positive immunostaining (HE×300). **C**, Anti PKIB antibody in cytoplasm of breast tumor showed positive immunostaining (HE×300).

Table I. Clinicopathological features of PKIB and pAkt positive or negative patients.

Pathological features	PKIB+	PKIB-	χ^2, p
pAkt+	20 (74.1%)	7 (25.9%)	12.790, 0.000
pAkt-	44 (36.4%)	77 (63.6%)	
Histopathological type			6.215, 0.102
Hard cancer	31 (48.4%)	46 (54.8%)	
Papillary adenocarcinoma	11 (17.2%)	18 (21.4%)	
Solid cancer	11 (17.2%)	16 (19.0%)	
Other	11 (17.2%)	4 (4.8%)	
Tumor diameter			0.606, 0.436
≤2.0 cm	34 (53.1%)	50 (59.5%)	
>2.0 cm	30 (46.9%)	34 (40.5%)	
Lymphatic metastasis			1.549, 0.213
LN+	22 (34.4%)	21 (25.0%)	
LN-	42 (65.6%)	63 (75.0%)	
Tumor stage			1.489, 0.685
I	30 (46.9%)	44 (52.4%)	
II	26 (40.6%)	31 (36.9%)	
III	7 (11.0%)	6 (7.2%)	
IV	1 (1.6%)	3 (3.6%)	
Preoperative adjuvant chemotherapy	7 (11.0%)	8 (9.5%)	0.080, 0.778

Note: PKIB (cAMP dependent protein kinase inhibitor B), pAkt (Phosphorylation Akt), LN (Lymph node).

The immunostaining of pAkt in 14 cases was homogeneous and obvious. In addition, the positive reaction was confined to the cytoplasm. PAkt in the 13 cases of tissue samples were found to have immune staining, which was negative staining in the majority lung cancer tissues, including epithelial cells, adjacent tissues and infiltrating inflammatory cells, as shown in Table I.

Expression of PKIB Was Significantly Associated with Three Negative Breast Cancer Subtypes

According to the expression of ER, PR and HER2 in tumor cells, 148 cases were divided into four groups: luminal A type (73 cases, 49.3%), luminal B type (30 cases, 20.3%), HER2+/ER- type (20 cases, 13.5%) and TNBC (25 cases, 16.9%). Table II showed the correlation between the positive expression rates of PKIB and pAkt in patients with primary tumors and breast cancer by immunohistochemistry. The correlation between PKIB and immunohistochemical subtypes was studied, and we found that the expression of PKIB was significantly associated with three negative breast cancer (p<0.05). In contrast, the expression of PKIB and pAkt were not significantly different among other three groups.

Discussion

PKIB is considered to have one of the regulatory factors mediating PKA signaling pathway[18]. Therefore, the overexpression of PKIB as

Table II. Correlation between PKIB and pAkt and IHC subtypes.

IHC Subtypes	PKIB expression on 148 cases			pAkt expression on 148 cases		
	PKIB+ (n = 64)	PKIB- (n = 84)	Association analysis χ^2, p	pAkt+ (n = 27)	pAkt- (n = 121)	Association analysis χ^2, p
Luminal A	25	48	20.614, 0.000	12	61	2.728, 0.435
Luminal B	10	20		6	24	
HER2/ER−	8	12		6	14	
TNBC	21	4		3	22	

Note: PKIB, PKIB (cAMP dependent protein kinase inhibitor B), pAkt (Phosphorylation Akt), IHC (Immuno histochemistry); Luminal A, ER+ and/or PR+, HER2−; Luminal B, ER+ and/or PR+, HER2+; HER2/ER-, ER-, PR-, and HER2+; TNBC, ER-, PR-, and HER2-.

a malignant phenotype predicts poor prognosis in patients with prostate cancer. PKIB is thought to promote the occurrence and development of prostate cancer through the phosphorylation of Akt in PKA and Akt pathway[19]. Although the hormone therapy is an ideal method for the treatment of breast cancer, the PKA is closely correlated with the endocrine therapy resistance when tamoxifen is used as the most critical prognostic factor in the treatment of breast cancer. In addition, the activation of the PKA signaling pathway is considered to be the basis of drug resistance when the tamoxifen is used as adjuvant chemotherapy for HER2 positive breast cancer. Other researches suggested that the inhibition of PKA activity can significantly reduce the expression level of HER2 protein in the mammary epithelial cells of tumor suppressor gene p53 inactivation[20]. The activation of Akt at downstream of HER2 pathway in breast cancer plays an important role in inhibiting the effect of endocrine therapy. Hence, the pAkt may be a valuable predictor of resistance of endocrine therapy in breast cancer, and it may also be a potential target for inhibiting Akt pathway and promoting endocrine therapy in breast cancer treatment[21]. Previous papers reported that the Akt activation or phosphorylation was important in developing the breast cancer[22]. This investigation confirmed significant correlation between the expression of PKIB and pAkt in tumor cytoplasm. These findings suggested that PKIB may participate in the regulation of Akt pathway through activation or phosphorylation of Akt. Compared with the prognosis of patients with luminal type A tumors, the progression-free survival and total survival time of TNBC patients were suggested to be shorter[23]. Another study found that activation of Akt pathway was more likely to occur in TNBC tissues[24]. In this study, the immunohistochemical staining of breast tumor tissue samples from 148 cases of primary breast cancer patients confirmed that the overexpression of PKIB was present in TNBC and there was a significant correlation between PKIB expression and pAkt. These results suggested that the overexpression of PKIB was associated with the development of breast cancer, and the expression of PKIB and pAkt can be detected in the breast cancer patients or as prognostic indicators. However, the continued expression of PKIB in breast tumors and other possible features, for example, the mechanism of overexpression of PKIB in TNBC is still unclear.

Conclusions

The immunohistochemical analysis confirmed that the expression of PKIB in tumor cytoplasm was closely associated with pAkt and TNBC. PKIB may promote the development of breast cancer by regulating Akt signaling pathway and it is a promising therapeutic target in the diagnosis and treatment of breast cancer, especially in TNBC.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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