Original Article

Increased Expression of β-klotho is Associated with Axillary Lymph Node Metastasis in Breast Cancer: An Immunohistological Study

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Objective: Breast cancer is a major cause of death in women worldwide including Thailand. The subject of molecular levels related with carcinogenesis and metastasis of breast cancer is poorly understood. The present study aimed to determine the expression of β-klotho (KLB) protein in breast cancer and its relation to axillaries lymph node metastasis.

Materials and Methods: KLB expression in 53 paraffin-embedded sections (34 breast cancer and 19 normal tissues) was determined by the immunohistochemistry method using monoclonal antibody specific to KLB protein. The clinico-pathological data of patients were also collected and analyzed with the laboratory data.

Results: The result showed a significant higher KLB expression in breast cancer compared to normal breast tissue (p=0.038). KLB expression was more frequently found in breast cancer tissue with metastasis to axillaries lymph node (43.75%, 16 of 34 cases) compared to breast cancer tissue without metastasis to axillaries lymph node (5.56%, 18 of 34 cases) and normal breast tissue (0%, 19 cases). Higher KLB expression was found in breast cancer with axillaries lymph node metastasis when compared to those without axillaries lymph node metastasis using a cut off value of immunohistochemistry index of 1 or more (p=0.015). In addition, the high levels of KLB expression (IHC index of 1 or more) can be a marker for axillaries lymph node metastasis (p=0.024, Exp (B)=0.032).

Conclusion: The present research was the first study in Thailand showing KLB protein overexpression in breast cancer, especially in breast cancer cases with axillaries lymph node metastasis. The result suggests the role of KLB protein in the process of cancer invasion and metastasis. Moreover, KLB protein overexpression could be a potential biomarker for breast cancer metastasis.

Keywords: β-Klotho, KLB, Axillaries lymph node, Metastasis, Breast cancer

J Med Assoc Thai 2019;102(10):1060-4
Website: http://www.jmatonline.com
Received 18 Mar 2019 | Revised 6 Jun 2019 | Accepted 7 Jun 2019

In recent decades, breast cancer incidence and mortality among women have gradually increased worldwide, including Thailand[1]. The research data extracted from the National Cancer Registry of Thailand 2011 to 2012 indicated a high incidence of breast cancer in the age group of 40 and older[2].

The most common breast cancer type for Thai patients was an infiltrating duct carcinoma with regional lymph node metastasis[2]. Metastasis is a final progression of solid cancer. This involves tumor cell intravasation, circulation, extravasation, angiogenesis, and continued growth in other organs and tissue[3]. The complexity of the molecular mechanism under breast cancer metastasis has been unclear. Therefore, a better understanding of the molecular regulation involving breast cancer progression may help to discover effective molecular markers to evaluate diagnosis and prognosis. These will help increase the success rate of therapy with lower mortality[4] because tumor metastasis can lead to poor chances of survival for patients[5].

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How to cite this article: Kotepui K, Kotepui M, Piwkham D, Songsri A, Charoenkijkajorn L, Sattaso A, et al. Increased Expression of β-klotho is Associated with Axillary Lymph Node Metastasis in Breast Cancer: An Immunohistological Study. J Med Assoc Thai 2019; 102:1060-4.

The β-klotho (KLB) gene family is associated with regulating lipid and glucose metabolism in the liver through interactions with fibroblast growth factor receptors (FGFRs)[6]. KLB is a single-pass transmembrane protein of 1,043 amino acids in length that share 41.2% homology with klotho α (KLA), which is located on 4p14[7]. KLB is primarily expressed in the liver, prostate, and kidneys[8]. Normally, KLB regulates bile acid homeostasis with an absence of KLB protecting against gallstones as shown in KLB-KO mice[9]. A recent study found that higher KLB expression in hepatocellular carcinoma correlated with cancer progression[10]. However, the KLB expression in breast cancer is not well understood. The present study aimed to determine the KLB expression in breast cancer. Moreover, KLB expression related to axillaries lymph node metastasis was also examined.

Materials and Methods

Human subjects and tissue specimens

The invasive ductal breast cancer and normal breast tissue were obtained from the Department of Pathology, Hatyai Hospital, Songkhla Province between January and December 2017. These cancers and normal tissue were previously confirmed as being cancerous with axillaries lymph node metastasis, breast cancer without axillaries lymph node metastasis, and normal breast tissue. The exclusion criteria were patients who had received prior radiotherapy or neoadjuvant therapies. Clinico-pathological data regarding age at diagnosis, type of tissue, grade of tumor, and regional lymph node status were also collected. The grading standard was used to assign scores of histological grades of breast cancer. Grade I consisted of well-differentiated tumors, grade II moderately differentiated tumors, and grade III poorly differentiated tumors. The present study was performed under a protocol approved by The Ethics Committee of Hatyai Hospital and the Ethical Clearance Committee on Human Rights related to research involving human subjects of Walailak University.

Immunohistochemistry

KLB expressions in breast tissue were determined using standard immunohistochemistry protocols. Briefly, the paraffin section was comprised of deparaffinized and hydrated, and the endogenous peroxidase was blocked with H_2O_2. After blocking with fetal bovine serum, the sections were incubated with 1:400 of KLB antibody at room temperature overnight. The secondary antibodies HRP conjugate and diaminobenzidine (DAB) were then added to the sections. The peroxidase activity was visualized by microscopic examination with at least two examiners per section. KLB positivity and intensity for each slide were examined by two reviewers. In case of different decisions by these two reviewers, a third reviewer expertise in the field was requested to confirm the KLB expression. The frequency of KLB positive cells was semi-quantitatively scored on the basis of the percentage of positive cells as 0%=negative, 1% to 25%=+1, 26% to 50%=+2, and more than 50%=+3. The intensity of KLB expression was scored as weak=1, moderate=2, and strong=3. The average KLB expression for each section was calculated as intensity multiplied by frequency and categorized as low (less than 1) or high (1 or more) KLB expression.

Statistical analysis

Statistical analyses were performed using the computer program Prism (GraphPad Software, La Jolla, CA) with a p-value of less than 0.05 being considered as statistically significant. Student's t-test were conducted to compare the KLB expression between breast cancer to normal breast and compare the KLB expression in axillaries lymph node positive to axillaries lymph node negative. KLB expressions in association with clinic-pathological data were determined by the Fisher's exact test. The potential value of KLB as a breast cancer biomarker between IHC index (less than 1 and 1 or more) and axillaries lymph node status was further evaluated by binary logistic regression analysis.

Results

The characteristics of breast tissue

Thirty-four cases of invasive ductal breast cancer and 19 normal breast tissue samples were collected. Among breast cancer cases, three cases (8.8%) were Grade I tumors, 15 (44.1%) were Grade II, and 16 (47.1%) were Grade III. According to axillaries lymph node status, 16 cases (47.1%) were lymph node positive and 18 (52.9%) were lymph node negative (Table 1). Fisher's exact test was performed to determine the correlation of the KLB expression level and clinic-histopathological features (age, type of tissues, tumor grade, and lymph node status (Table 2). No correlation between age at diagnosis and expression of KLB was found ($p>0.05$).

KLB positive cell staining in breast cancer and normal tissue

KLB expression was determined in 34 invasive

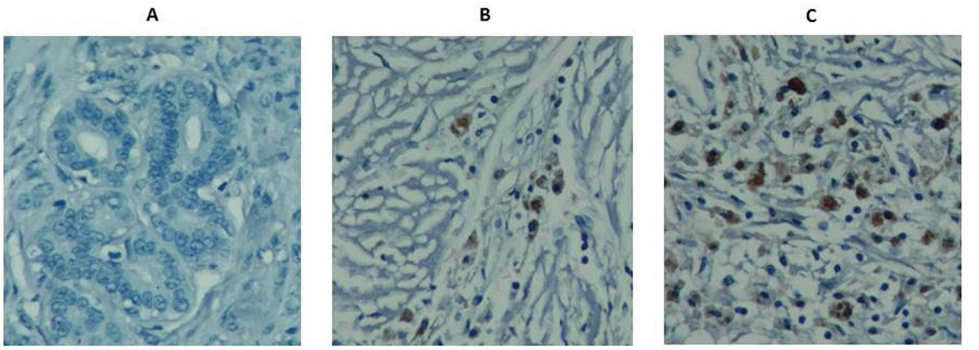

Figure 1. KLB expression in breast tissue. The immunohistochemical staining of KLB in normal breast tissue (A), breast cancer tissue without metastasis to axillaries lymph node (B) and breast cancer tissue with metastasis to axillaries lymph node (C), 40× magnification.

Table 1. Clinico-pathological characteristic of patients

Characteristic	Number (%)
Age (years), Mean±SD	44.5 (12.1)
Type of tissues	
Tumor	34 (64.2)
Normal	19 (35.8)
Lymph node status	
Positive	16 (47.1)
Negative	18 (52.9)
Differentiation of tumor	
Well	3 (8.8)
Moderate	15 (44.1)
Poor	16 (47.1)

SD=standard deviation

Table 2. Clinico-pathological characteristics of patients analyzed by KLB expression

Clinico-pathological characteristics	IHC index, n (%)		p-value[a]
	IHC index <1	IHC index ≥1	
Age (years)			1.000
<45	6 (75.0)	2 (25.0)	
≥45	20 (76.9)	6 (23.1)	
Type of tissues			0.038*
Tumor	26 (76.5)	8 (23.5)	
Normal	19 (100)	0 (0.0)	
Lymph node status			0.015*
Positive	9 (56.3)	7 (43.7)	
Negative	17 (94.4)	1 (5.6)	
Differentiation			0.360
Well	2 (66.7)	1 (33.3)	
Moderate	10 (66.7)	5 (33.3)	
Poor	14 (87.5)	2 (12.5)	

IHC=immunohistochemistry index

[a] p-value by Fisher's exact test, * Statistically significant

ductal breast cancer patients' tissue samples, of which 16 cases with positive and 18 cases with negative lymph node metastasis. Eight of the breast cancer cases (23.53%) showed positive cell staining for KLB. Among lymph node positive cases, seven cases (43.75%) showed some degree of positive tumor cell staining. Meanwhile, of the lymph node negative cases, only one showed some degree of positive tumor cell staining (5.56%) as shown in Figure 1. No positive staining (0%, 0/19) for KLB was found in normal breast tissue.

KLB expression level in breast cancer and normal tissue

KLB expression level was evaluated from immunohistochemistry index (IHC; intensity × percent of positive staining). Distribution of KLB expression levels in individual breast cancer cases compared to normal breast tissue is shown in Figure 2. KLB expression level in breast cancer tissue was significantly higher than those of normal breast tissue (p=0.038).

KLB expression level in axillaries lymph node positive and axillaries lymph node negative breast cancer

High levels of KLB expression (IHC index of 1 or more) was correlated with the type of tissues examined (p=0.038) and axillaries lymph node status (p=0.015). High levels of KLB expression was not correlated with the differentiation of tumors (p=0.360).

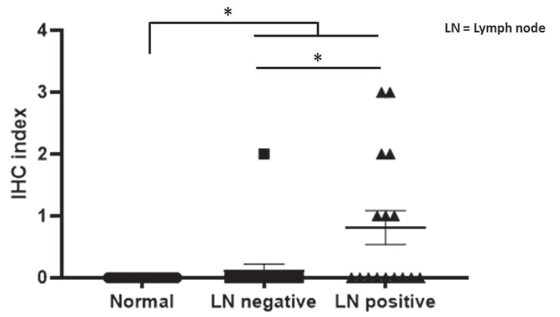

Figure 2. Immunohistochemistry of KLB was performed in 34 cases of breast cancer patient tissues.

* p<0.05, ** p<0.01

Table 3. Binary logistic regression analysis of IHC index and axillaries lymph node metastasis

	B	SE	Sig	Exp (B)
Constant	–4.528	2.177	0.038	0.011
IHC index	2.582	1.146	0.024	13.22

The potential value of KLB as a metastasis breast cancer

The potential value of KLB as a breast cancer biomarker was further evaluated by binary logistic regression analysis. The result showed that high levels of KLB expression (IHC index of 1 or more) can be a marker for axillaries lymph node metastasis (p=0.024, Exp (B)=0.032) (Table 3).

Discussion

Tumor invasion and metastasis is the main cause of death from breast cancer[11]. In the present study, high KLB expression was found in the breast cancer tissue, especially breast cancer cases with axillaries lymph node metastasis. This observation indicated the possible relation of KLB with carcinogenesis and progressiveness of breast cancer. A similar observation reported that KLB activated FGFR4 activities in hepatocellular carcinoma progression (Poh et al[10], 2012). There are reports that overexpression of FGFR4 promotes cell cycle progression in several cancers[12] including breast cancer[13]. KLB is a co-receptor of FGFR4[14], so increasing of KLB expression in breast cancer might activate FGFR4 downstream signaling. However, the authors were currently unable to determine the cause of high expression of KLB in breast cancer. On the other hand, the result of the present study was not consistent with a report showing KLB inhibited cell growth and progression of prostate cancer through inactivation of the extracellular-signal-regulated kinase (ERK) pathway[15]. This suggested that KLB has diverse functions in different cancer types.

Axillaries lymph node metastasis is one of the most important prognostic factors in breast cancer progression. Poor prognosis of breast cancer is related to the high number of tumor-positive lymph nodes[16]. The present study found significantly higher KLB expression in breast cancer related to cancer infiltration of axillary lymph nodes. This finding suggests that KLB is likely to play a role in breast tumor progression and metastasis. However, the result of the present study was in contrast with a previous study indicating that KLB mRNA level expression was not associated with axillaries node status in breast cancer[17]. Moreover, the result of the present study was in contrast with the most recent study indicating that KLB expression was negatively associated with lymph node metastasis[18]. The authors suggested that this phenomenon might be due to the fact that the authors used a different of sample group and another reason was the modifications of post-translation steps such as phosphorylation, acetylatylation, glycosylation, etc., may have increased protein since post-transcriptional processes are keys to the final synthesis of the native protein[19].

The present study had a limitation as only a small number of breast cancerous and normal tissue were collected. Further studies with a larger number of breast tissue and the molecular underling of axillary lymph node metastasis in breast cancer should be performed.

Conclusion

In conclusion, the result of the present study indicated that KLB expression was frequently found in breast cancer, especially breast cancer with lymph node positive. This might suggest its role in breast cancer progression and metastasis.

What is already known on this topic?

Research has shown that Klotho beta (KLB) gene plays a key role in the inhibition of cancer development and metastasis. However, the study of molecular levels related with carcinogenesis and metastasis of breast cancer is still poorly understood.

What this study adds?

Most of breast cancer patients' tissues expressed KLB more intensely than the normal breast. In addition, KLB expression was more frequently found

in breast cancer tissue with metastasis to axillaries lymph node compared to those without metastasis to axillaries lymph node and normal breast tissue.

Acknowledgement

The present study was partly supported by the operating research grants from Walailak University. The authors would like to thank the staff of the Department of Pathology, Hatyai Hospital, Songkhla, Thailand for their support and assistance in providing samples to conduct this project.

Data availability

The datasets used during the current study are available from the corresponding author upon reasonable request.

Funding disclosure

This study was supported by funds from Walailak University.

Conflicts of interest

The authors declare no conflict of interest.

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