Tissue Expression Of LPHN3 in Breast Cancer: An Immunohistochemistry Method

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Abstract

Objective: Breast cancer is one of the most important public health problems among women worldwide. It is a major cause of morbidity especially among women in developing countries including Thailand. The purpose of this study was to study the expression of LPHN3 protein in normal breast tissue compared to breast cancer tissue. Methods: We had studied the expression of LPHN3 in 65 breast tissues using an immunohistochemistry method. The association between LPHN3 expression and breast cancer metastasis to nearby axillary lymph nodes was also examined. Results: Among the 65 breast cancer and normal breast tissues examined, LPHN3 expression with an immunohistochemistry index (IHC index) greater than 4 was more frequently found in breast cancer tissues than in normal breast tissues (P-value = 0.001, OR (95% CI) = 7.04 (2.16-23)). Moreover, a high expression of LPHN3 (IHC index > 4) was more frequently found in breast cancer tissues with negative axillary lymph nodes than in those with positive ones (P-value = 0.038, OR (95% CI) = 0.25 (0.07-0.96)). LPHN3 protein might be a new metastasis suppressor gene in breast cancer and a marker for breast cancer metastasis prevention. Conclusions: The present study indicated that a decrease of LPHN3 protein expression in breast cancer tissue might be a marker indicating the aggressiveness of breast cancer. These results also suggested that a decrease of LPHN3 expression could be functionally involved in breast cancer progression and metastasis.

Keywords: LPHN3- breast cancer- lymph node metastasis

Introduction

Breast cancer is the most common cancer among women worldwide affecting 2.1 million persons annually, and it is the leading cause of cancer death among women (WHO). In Thailand, breast cancer is the third most common cancer with 19,510 new cases in 2018 of which 5,902 cases resulted in death (Bray et al., 2018). The data from a study at Songklanagarind Hospital in Southern Thailand estimated 671 breast cancer cases in 2018 (Prechawittayakul, 2009). Breast cancer is still frequently diagnosed at an advanced stage, and the study of protein expression as a biomarker is still elusive. 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 (Wang, 2017) because tumor metastasis can lead to poor chances of survival for patients (Jemal et al., 2008). An infiltrating duct carcinoma with regional lymph node metastasis is the most common breast cancer type among Thai patients (Kotepui and Chuepeerach, 2014). Metastasis is the final progression of solid cancer. This involves tumor cell intravasation, circulation, extravasation, angiogenesis, and continued growth in other organs and tissues (Valastyan and Weinberg, 2011). The majority of cancers begin developing metastatic clones and spreading via lymphatic vessels to other lymph nodes and other organs. The detection of tumor with lymph node metastases contribute to major prognostic implications and the selection of adjuvant therapies for improving patient survival (Wu et al., 2014).

LPHN3 (the latrophilin 3 gene) is a member of the G-protein coupled receptor (GPCR) family with a large extracellular and intracellular domain, containing several cell adhesion modules such as cadherin, IgG, laminin A, thrombospondin type 1, galactose lectin, EGF, and transmembrane segments that may be involved in intracellular signaling during cell-to-cell adhesion (Wu et al., 2014). A previous study indicated that LPHN3 was up-regulated significantly in a transgenic mice model that over-expressed myocilin (Paper et al., 2008). Altered LPHN3 expressions in brain ischemia have been observed (Bin Sun et al., 2002). Mice lacking the LPHN3
expression resulted in attention deficit-hyperactivity disorder (ADHD), the most common psychiatric disorder in childhood and adolescence (Wallis et al., 2012). Nevertheless, the study of LPHN3 in human cancer is poorly understood.

Increased mRNA expression of LPHN3 and MMP13 was significantly associated with axillary node metastasis assessed by RT-PCR (Kotepui et al., 2012). However, the LPHN3 expression at the protein level in breast cancer is still unelucidated. The present study aimed to evaluate the LPHN3 expression in breast cancer. Moreover, LPHN3 expression related to axillary lymph node metastasis was also examined.

Materials and Methods

Human subjects and tissue specimens

Tissue samples, including invasive ductal breast cancer and normal breast tissues that were diagnosed and surgically treated, were obtained from the Department of Pathology, Hatyai Hospital, Songkhla Province, between January to December 2017. The female patients had not received prior radiotherapy or neoadjuvant therapies before recruiting the tissues. The patient characteristics include age at diagnosis, type of tissue, grade of tumor, and regional lymph node status. Grading standard was commonly used to assign the scores of histological grades of breast cancer. Grade I is well-differentiated tumors, Grade II is moderately differentiated tumors, and Grade III is poorly differentiated tumors. This study was performed under a protocol approved by the Ethic Committee of Hatyai Hospital and the Ethical Clearance Committee on Human Rights Related to Researches Involving (WU-EC-MT-2-045/59). Informed consent was not obtained from participants, but patient records/information was anonymized and de-identified prior to this analysis. The name and Hospital Number (HN) of patients were not revealed.

Immunohistochemistry (IHC)

LPHN3 was detected using standard immunohistochemistry protocols. Specifically, the paraffin sections were deparaffinized and hydrated, and then the endogenous peroxidase was blocked with H2O2. After blocking with normal serum, the sections were incubated with 1:100 of LPHN3 Polyclonal Antibody (ab150794, Thermo Fisher Scientific Inc, MA, USA) at room temperature for overnight, and secondary Ab and peroxidase activity was visualized with a diaminobenzidine (DAB) solution. The frequency of LPHN3 positive cells was semi-quantitatively scored on the basis of the percentage of positive cells, where 0% = negative, 1-25% = +1, 26-50% = +2, and > 50% = +3. The intensity of the LPHN3 expression was scored as weak = 1, moderate = 2, and strong = 3. The average LPHN3 expression of each section was calculated as intensity multiplied by frequency and categorized as low (≤ 4) or high (> 4).

Statistical analysis

Statistical analyses were performed using SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA), and the computer program Prism (GraphPad Software, La Jolla, CA). Student’s t-test was used for comparison between two groups. A P-value of < 0.05 was considered statistically significant. LPHN3 expression was evaluated for association with clinicopathological findings using the Chi2-test (*P < 0.05, **P < 0.01, and ***P < 0.001).

Results

Characteristics of included breast cancer cases

Among the 65 breast tissues retrieved from Hatyai Hospital, 22 cases (33.8%) were normal breast tissues whereas 43 cases (66.2%) were breast cancer tissues. Among the 43 breast cancer tissues, 18 cases (41.9%) were positive axillary lymph nodes whereas 25 cases (58.1%) were positive axillary lymph nodes whereas 25 cases (58.1%)

Table 1. Patient Characteristics Included in the Study

| Patient characteristics | Frequency (%) |
|-------------------------|---------------|
| Age (mean±SD)           | 46.8±3.04     |
| Breast tissue           |               |
| Normal                  | 22 (33.8)     |
| Cancer                  | 43 (66.2)     |
| Tumor size (mean±SD)    | 3.04±1.55     |
| Grade                   |               |
| Grade I                 | 4 (10.3)      |
| Grade II                | 23 (59)       |
| Grade III               | 12 (30.8)     |
| Lymph node status       |               |
| Negative                | 25 (58.1)     |
| Positive                | 18 (41.9)     |

Figure 1. Immunohistochemistry of Normal Breast Tissue (A), breast cancer tissue with negative lymph nodes (B), and breast cancer tissue with positive lymph nodes (C) (40X)
Tissue Expression of LPHN3 in Breast Cancer with Axillary Lymph Nodes Status Using an Immunohistochemistry Method

The LPHN3 expression in all the types of breast tissues is shown in Figure 1 and Table 2. The results showed that most of the normal breast tissues exhibited an IHC index at 4 (7 cases, 31.8%). Most of the breast cancer tissues with negative axillary lymph nodes exhibited an IHC index at 6 (15 cases, 60%). Most of the breast cancer tissues with positive axillary lymph nodes also exhibited an IHC index at 6 (7 cases, 38.9%).

Prognosis factor of LPHN3 expression in breast cancer tissues with positive axillary lymph nodes

The IHC index was categorized into > 4 or ≤ 4 based upon a mean IHC index (4.8). LPHN3 expression with an IHC index > 4 was more frequently found in breast cancer tissues with positive axillary lymph nodes than in those with positive ones (P-value = 0.038, OR (95% CI) = 0.25 (0.07-0.96)) (Table 3). Regression analysis of the LPHN3 expression with an IHC index > 4 indicated significance of LPHN3 expression as both a breast cancer prevention biomarker and also for anti-tumor metastasis to axillary lymph nodes (P-value = 0.001) (Table 4).

Discussion

The present study evaluated the LPHN3 expression in normal breast and cancer tissues. The results revealed that LPHN3 was generally expressed in both normal breast and cancer tissues with different intensities. LPHN3 was more highly expressed in breast cancer tissues than in normal tissues (P-value = 0.001, OR (95% CI) = 7.04 (2.16-23)). Moreover, LPHN3 expression with an IHC index > 4 was more frequently found in breast cancer tissues with negative axillary lymph nodes than in those with positive ones (P-value = 0.038, OR (95% CI) = 0.25 (0.07-0.96)) (Table 3). Regression analysis of the LPHN3 expression with an IHC index > 4 indicated significance of LPHN3 expression as both a breast cancer prevention biomarker and also for anti-tumor metastasis to axillary lymph nodes (P-value = 0.001) (Table 4).

Table 2. LPHN3 Expression in All the Types of Breast Tissues

| IHC index | Normal (%) | Breast cancer with negative axillary lymph nodes (%) | Breast cancer with positive axillary lymph nodes (%) | Total (%) |
|-----------|------------|-----------------------------------------------|-----------------------------------------------|----------|
| 1         | 4 (18.2)   | 1 (4)                                          | 2 (11.1)                                      | 7 (10.8) |
| 2         | 3 (13.6)   | 1 (4)                                          | 3 (16.7)                                      | 7 (10.8) |
| 3         | 3 (13.6)   | 0                                              | 1 (5.6)                                       | 4 (6.2)  |
| 4         | 7 (31.8)   | 3 (12)                                         | 3 (16.7)                                      | 13 (20)  |
| 6         | 3 (13.6)   | 15 (60)                                        | 7 (38.9)                                      | 25 (38.5)|
| 9         | 2 (9.1)    | 5 (20)                                         | 2 (11.1)                                      | 9 (13.8) |

P-value by Pearson Chi-Square

| LPHN3 expression | Breast tissues | P-value | OR (95% CI) |
|------------------|----------------|---------|-------------|
| Normal (%)       | Cancer (%)     |         |             |
| ≤ 4              | 17 (73.3)      | 14 (32.6) | 0.001       | 7.04 (2.16-23) |
| > 4              | 5 (22.7)       | 29 (67.4) |             |             |

IHC index

| Negative (%) | Positive (%) |
|--------------|--------------|
| ≤ 4          | 5 (20)       | 9 (50)      |
| > 4          | 20 (80)      | 9 (50)      |

P-value by Pearson Chi-Square

Table 4. Regression Analysis of LPHN3 Expression with IHC Index > 4

| LPHN3 expression as a breast cancer marker | B     | S.E.  | Wald | df  | Sig.  | Exp(B) |
|-------------------------------------------|-------|-------|------|-----|-------|--------|
| IHC index                                 | -1.952| 0.604 | 10.447| 1   | 0.001 | 0.142  |
| Constant                                  | 1.758 | 0.484 | 13.178| 1   | 0     | 5.8    |

LPHN3 expression as a breast cancer marker for tumor metastasis to axillary lymph nodes

| IHC index | B     | S.E.  | Wald | df  | Sig.  | Exp(B) |
|-----------|-------|-------|------|-----|-------|--------|
| ≤ 4       | 1.386 | 0.687 | 4.07 | 1   | 0.044 | 4      |
| > 4       | -0.799| 0.401 | 3.958| 1   | 0.047 | 0.45   |

P-value by Pearson Chi-Square
coupled receptor family associated with both ADHD genetic susceptibility and methylphenidate pharmacogenetics (Bruxel et al., 2015). High LPHN3 expression has been previously reported in a transgenic mouse model that over-expressed myocilin and in mice after brain ischemia (Wu et al., 2014; Paper et al., 2008). Mice lacking the LPHN3 expression resulted in attention deficit-hyperactivity disorder (ADHD), the most common psychiatric disorder in childhood and adolescence (Wallis et al., 2012). It has also been demonstrated that LPHN3 activation in pancreatic islets reduces insulin secretion (Rothe J et al., 2019).

The increase of LPHN3 in breast cancer tissues was at significantly higher levels when compared to healthy breast tissues. It has been previously reported that primary breast tumors and MCF-7 cells expressed comparable amounts of LPHN3 (Yasinska et al., 2019). Increased mRNA expression of LPHN3 and MMP13 was significantly associated with axillary node metastasis assessed by RT-PCR (Kotepui et al., 2012). In addition, Jahn et al., (2016) found 1% mutation of LPHN3 in the usual ductal hyperplasia (UDH) of the breast. In contrast, our study revealed that the LPHN3 expression was more frequently found in breast cancer tissues with negative axillary lymph nodes than in those with positive ones.

In Thailand, the incidence rate of breast cancer varies geographically based upon diverse lifestyles, behaviors, and risk profiles of the northern, northeastern, central, and southern regions of Thailand (Jordan et al., 2009). For example, a previous study showed that tumors among Muslims were histologically homogeneous; whereas tumors among Buddhists exhibited heterogeneity, which may have genetic, biological, and management implications (Pang et al., 2018).

The intensity of LPHN3 immuno-reactivity was different according to the histological grading subtype of breast cancer. LPHN3 was highly expressed in moderately to poorly-differentiated carcinomas but down-regulated in well-differentiated tumors. Axillary lymph nodes are the most common initial site of metastatic disease (Woods et al., 2019). It is not only a marker of diagnosis at a later point in the natural history of breast cancer but also a marker of an aggressive phenotype (Jatop et al., 1999). Although the high expression of this protein was found in poor differentiation histological grade (8/12, 66.7%), the lower expression of this protein was also found in well differentiation histological grade (4/12, 33.3%). Nevertheless, the statistically significance of lower LPHN3 expression was not statistically significance (p=0.053). This might due to the low sample size evaluated in the present study. In this study, the association of low LPHN3 expression and lymphatic invasion was observed. Moreover, this study found that higher LPHN3 expression was not related to axillary lymph node metastasis.

In conclusion, our findings indicated that the decrease of the LPHN3 protein expression in breast cancer tissues may be a new important tumor marker and a new marker indicating aggressiveness of breast cancer. These results also suggested that LPHN3 could be functionally involved in breast cancer progression and metastasis. However, further studies need to examine the details of the underlying mechanism of low LPHN3 protein expression, which may influence the breast cancer metastasis phenotype.

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