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The Expression of ASPH in Breast Cancer

Zhao Xiaofeng[1, *, †], Qin Xuying[2, †], Yin Linlin[3], Chen Dong[4], Zheng Juan[1]

[1]Joint Laboratory for Translational Medicine Research, Liao Cheng People's Hospital, Liaocheng, China

[2]Department of Gynaecology and Obstetrics, Liao Cheng People's Hospital, Liaocheng, China

[3]Department of Hematology, The Fourth People's Hospital of Jinan, Jinan, China

[4]Department of Nursing, Taishan Nursing Vocational College, Tai'an, China

Email address:

maplelj@163.com (Zhao Xiaofeng)

[*]Corresponding author

†Zhao Xiaofeng and Qin Xuying are co-first authors.

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Abstract: Asparagine-betahydroxylase (ASPH) plays an important role in cell growth, differentiation, adhesion and migration. And ASPH is silent in adult tissues in normal. It expresses at very low levels or not expresses at all in most normal cells and tissues. High level of ASPH always be detected in aggressive tumor cells and it indicates worsened clinical prognosis. The expression of ASPH in paraffin-embedded tissue sections was detected by immunostaining. We analyzed the distributions and levels of ASPH by immunohistochemical staining in breast cancer. ASPH is expressed high in breast cancer. And we detected the expression of ASPH by Elisa in breast cancer cells (MCF7) and normal mammary epithelial cells (MCF10A). The level of ASPH is higher in MCF7 than MCF10A. In breast cancer, the ASPH positive cases are 15 of 34, and the positive rate is 44.1%. The higher grade cancer was associated with the higher levels of ASPH. And we found that the ASPH expression have relation to the expression of progesterone receptor (PR) and HER-2. Our study demonstrates that increased ASPH expression in breast cancer, and it has closed relation to the level of PR and HER-2. Therefore, ASPH may be closely related to the malignant degree and prognosis of breast cancer.

Keywords: ASPH, Breast Cancer, ER, PR, Her-2

1. Introduction

Asparagine-betahydroxylase (ASPH) is a deoxygenase dependent on alpha-ketoglutarate, which exists in mammalian cells during embryonic period [1, 2]. Its expression disappears in adulthood and is highly conservative. Professor JACK of Brown University found that ASPH was detected on the membranes of hepato cellular carcinoma and pancreatic cancer, with positive rates of 81.48% and 97.1% respectively, while normal cells were negative [3-5]. At the same time, ASPH was detected in the serum of patients with lung cancer, which could be used as a serum marker of lung cancer [6] and circASPH may contribute to the development of circRNA-based the therapeutic strategies for lung adenocarcinoma [7]. ASPH has a relative molecular weight of 86kD and 27 exons. It encodes four proteins: AAH, humbug, junctate and junctin. AAH and humbug proteins are the main expression products of ASPH gene [8, 9]. ASPH catalyzes the hydroxylation of beta-carbon atoms on the aspartic acid or asparagines residues of epidermal growth factor-like domains (such as Notch and Jagged) in specific proteins, and plays an important role in cell growth, differentiation, adhesion and migration [10]. The normal expression of ASPH in adults is "off" and only "open" when tumors occur. It is considered that the function of ASPH is related to the phenotype of malignant tumor [5, 11].

The incidence of breast cancer is the highest among female malignant tumors, and it shows a growing trend. Biomarkers play an important role in the patients with invasive breast cancer, such as oestrogen receptors (ERs), progesterone receptors (PRs) and human epidermal growth factor receptor 2 (HER-2). Accurate reporting of estrogen

receptor (ER), progesterone receptor (PR) and HER2 status in breast cancer is necessary for prognostic. The most recent clinical guidelines recommend testing of ER, PR, and HER2 for all primary invasive breast cancer cases and recurrent lesions [12]. The main clinical application of steroid hormone receptors, ER and PR is in selecting patients with invasive breast cancer for treatment with endocrine therapy. As predictive markers for endocrine therapy, ER and PR are used in the neoadjuvant, and advanced disease settings [13-15]. And several retrospective studies have shown that patients with ER or PR-containing tumours tend to have a better outcome than those lacking the receptors [16-18]. And the same to ER and PR, the main clinical use of HER2 measurement is in predicting the response to anti-HER2 therapy in the neoadjuvant, adjuvant and advanced disease settings [19].

Our study was to observe the expression of ASPH in breast cancer patients by immunohistochemistry and Elisa to observe the relationship between ASPH expression and ER, PR and HER2. And ASPH may be a new marker in breast cancer and it is predicted to be a new therapy for breast cancer.

2. Materials and Methods

2.1. Human Subjects

All tissues were derived from the Pathological Department of Liao Cheng People's Hospital past five years. There were 34 breast cancer patients, which classifies into 6 Luminal A, 12 Luminal B, 10 ERBB2+ and 6 Basal-like. The investigation was conducted and approved in accordance with the ethical standards of the Declaration of Helsinki, national and international guidelines and was approved by the Ethics Committee of Liao Cheng People's Hospital.

2.2. Immunohistochemistry

The breast cancer tissues were soaked in 10% formaldehyde solution for 24 hours, after 60% alcohol for 60 minutes, 70% alcohol for 90 minutes, 85% alcohol for 90 minutes, 90% alcohol for 90 minutes, 95% alcohol twice for 90 minutes each, 100% alcohol twice for 70 minutes and 90 minutes respectively, then Xylene twice for 60 minutes each and Xylene for 90 minutes. Afterwards, the tissues were immersed in paraffin four times for 45 minutes each at 62°C, embedded and sectioned with HistoCore BLOCUT slicer. The thickness of pathological sections were 4μm. The immunohistochemical procedure is as follows:

De-paraffinize by Xylene (15 minutes) x3, followed by Ethanol gradient (100%, 100%, 95%, 90%, 80%, 70%, 60%, 1 minute each). Preheat 1L of diluted Antigen Unmasking Solution (VectorLabH-3300) in a Nordic Ware Pressure Cooker using a microwave oven for 6 minutes. Perform endogenous peroxidase blocking by placing slides in MeOH 200ml +30% Hydrogen Peroxide 13.5ml for 30 minutes in a plastic slide container. Perform antigen blocking at room temperature for 30 minutes using blocking buffer from Elite ABC Kit (Vector Lab PK-6102). Dilute FB-50 (Provided by Brown University, USA) at 1:10,000 in blocking buffer from Elite ABC Kit (Vector Lab PK-6102). Incubate with just enough diluted FB-50 antibody to cover the slide in a sealed container at 4°C overnight to minimize evaporation. Incubate with secondary antibody at room temperature for 30 minutes using diluted secondary antibody from Elite ABC Kit (Vector Lab PK-6102). Perform peroxidase-labeling using dilated ABC reagent from Elite ABC Kit (Vector Lab PK-6102). Color development using DAB Peroxidase Substrate (Vector Lab SK-4103) for 15-120 seconds, depending on sample tissues. Stop the reaction by placing the slides in 1×Phosphate Buffered Saline. Counterstain by applying Hematoxylin (Vector Lab H-3401) directly on top of slides for 30 seconds. Dehydrate using reversed Ethanol gradient (60%, 70%, 80%, 90%, 95%, 100%, 100%, 1 minute each) and Xylene (for 15 minutes). Mount using a drop of Aqueous Mounting Medium (Vector Lab H-5501) and cover with a coverslip.

2.3. Cell Culture

Human breast carcinoma cell lines MCF-7 (ATCC, HTB-22) were cultured under the conditions recommended by the DMEM high glucose medium (GIBCO) supplemented 10% FBS and 1% Penicillin-Streptomycin, and Normal epithelial cells of the human breast MCF-10A cells (ATCC) were cultured in DMEM/Ham'sF-12 (GIBCO) supplemented with 5% chelex-treated horse serum, 10μg/ml insulin, 20ng/ml epidermal growth factor (EGF), 100ng/ml cholera toxin and 0.5μg/ml hydrocortisone.

2.4. Elisa for ASPH

The cells were washed gently with cold PBS and separated with trypsin, centrifuged for 5 minutes at 1,000×g. The cells were rinsed with cold PBS three times. The cells were resuspended in fresh lysis buffer at 107 cells/ ml. The cell fragments were removed after centrifugation at 1,500×g for 10 minutes at 4°C. Prepare 7wells of standard solution and 1 well of blank solution. Add standard, blank, and sample 100μL diluents each to corresponding wells. Incubate at 37°C for 1 hour. Add Detection Reagent A working solution 100μL to each well, cover with sealing plate, and incubate at 37°C for 1 hour. Suction 1×washing solution 350μL to each well with spray bottle, multi-channel pipette, tube distributor or automatic cleaning machine, stand for 1~2 minutes. Place the plate on blotting paper to remove any remaining liquid from all the holes. Three times in total. Add Detection Reagent B working solution 100μL to each well, cover with sealing plate, and incubate at 37°C for 30 minutes. Add 90μL Substrate Solution to each well. Incubate at 37°C for 10-20 minutes. From light protection. When the Substrate Solution is added, the liquid turns blue. Add 50μL Stop Solution to each well. Then, the microplate reader was run and immediately measured at 450nm.

2.5. Statistics

SPSS 19.0 software was used for statistical analysis. The expression of immunohistochemistry was analyzed by ANOVA. The correlation of ASPH with clinicopatholigical characteristics was assessed by the Chi-square test. P values less than 0.05 was considered statistically significant.

3. Results

3.1. ASPH Is High Level Expression in Breast Cancer

Formalin-fixed paraffin embedded histological sections of 34 breast cancer patients were immunostained to detect ASPH. In breast cancer, the positive cases are 15 of 34, and the positive rate is 44.1%. ASPH protein is highly expressed in breast cancer. (Figure 1).

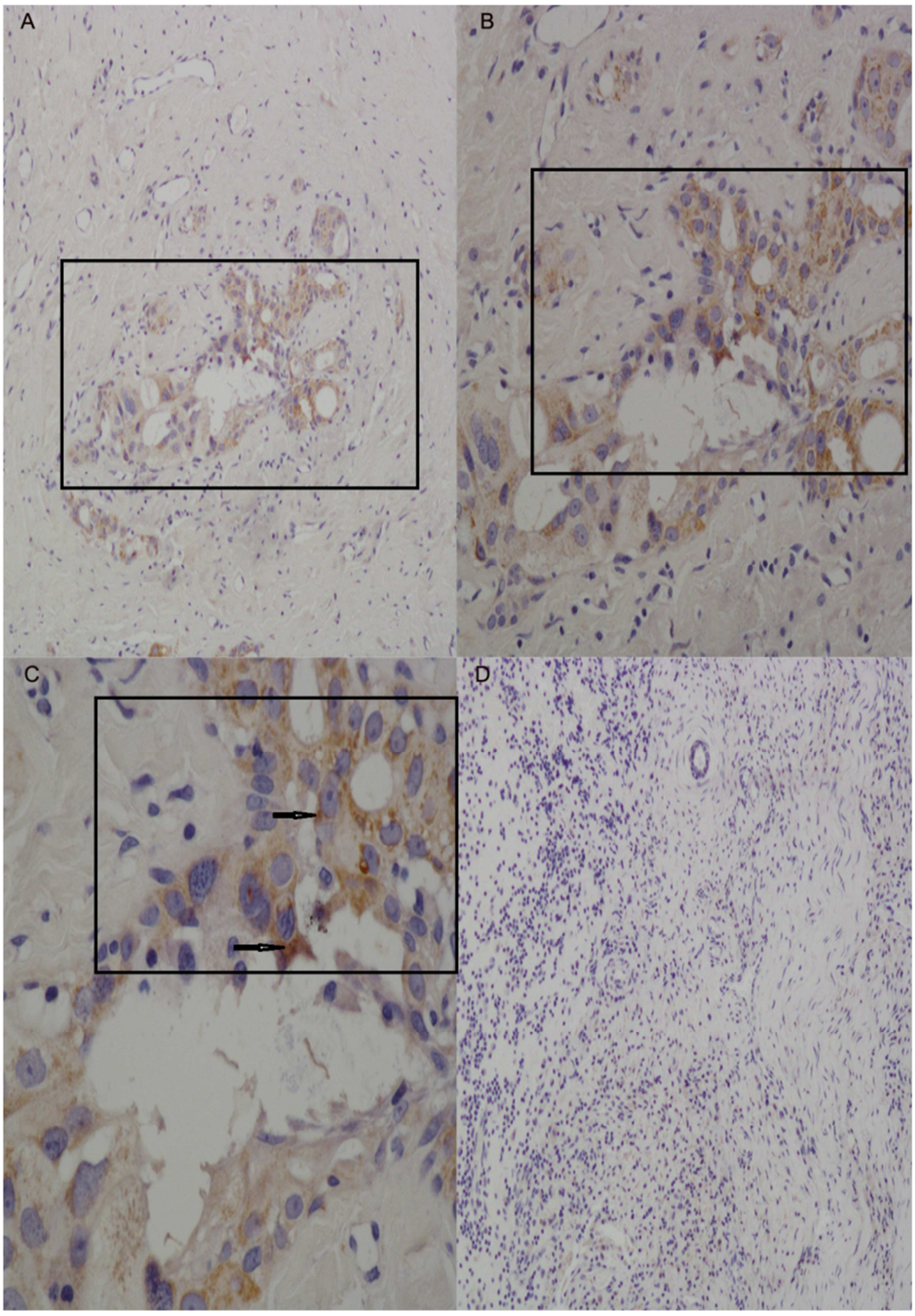

Figure 1. *ASPH expression in breast cancer is positive and the expression in Cystic changes of the breast is negative. (A) ASPH expression in breast cancer (10×); (B) (20×) and (C) (40×) are the ASPH expression in breast cancer; (D) ASPH expression in Cystic changes of the breast (10×). The arrow is positive.*

3.2. Elisa for ASPH

The expression of ASPH was detected in MCF7 and MCF10A. The results showed that the expression of ASPH in MCF7 is much more than in MCF10A. The P value is 0.0002 (Figure 2). This suggests that ASPH is highly expressed in breast cancer cells compared with normal breast cells.

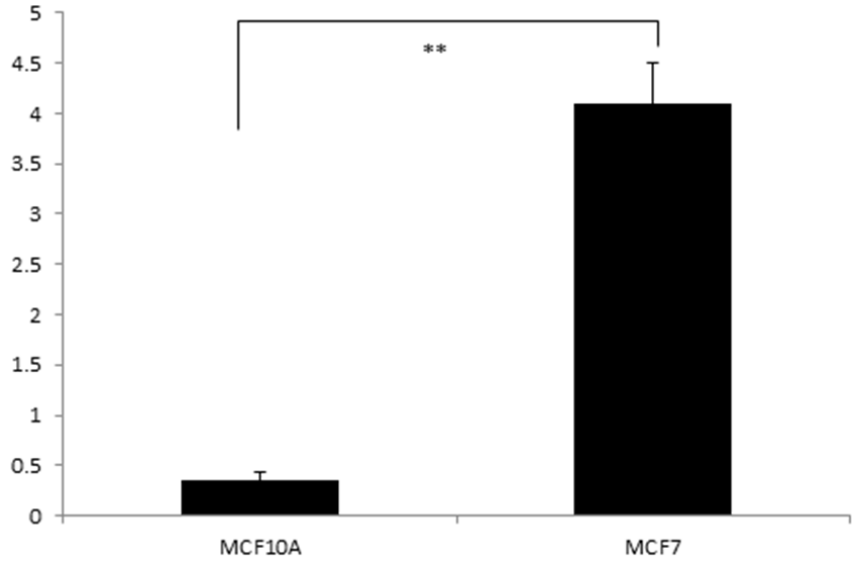

Figure 2. *The expression of ASPH in MCF7 is higher than MCF10A (P <0.001).*

3.3. ASPH Is Closely Related to the Expression of PR and HER-2

The expression of ASPH protein was not correlated with tumor size, lymph node metastasis, clinical stage, WHO grade, ER and Ki67 (all P>0.05), but was correlated with the expression of PR and HER2 (all P<0.05). Chi-square test was used to evaluate the correlation between ASPH with PR and HER-2. P values less than 0.05 was considered statistically significant. P value of ASPH and PR is 0.032, and P value of ASPH and HER-2 is 0.036 (Table 1).

Table 1. *Correlations of ASPH expression with clinicopathological features of breast cancer.*

Variable	ASPH		2	P value
	Positive	Negative		
Tumor size			0.295	0.863
T1	5	6		
T2	5	9		
T3+T4	4	5		
Lymphatic metastasis			0.647	0.212
N0	6	7		
N1	1	7		
N2	4	5		
N3	3	1		
Clinical stage			1.191	0.551
I	1	4		
II	6	7		
III	7	9		
WHO grade			0.008	0.93
I+II	5	8		
III	8	12		
ER			2.238	0.135
Positive	8	7		
Negative	5	13		
PR			3.993	0.032
Positive	10	7		
Negative	3	13		
Her2			3.481	0.036
Positive	8	4		
Negative	6	16		
P53			0.787	0.364
Positive	1	5		
Negative	13	15		
Ki67			0.596	0.44
High	6	6		
Low	8	14		

Table 2. *The number of cases of breast cancer pathological classification.*

Molecular type	number
Luminal A	6
Luminal B	12
ERBB2+	10
Basal-like	6

4. Conclusion

The expression of ASPH is high in breast cancer. The ASPH positive cases are 15 of 34, and the positive rate is 44.1%. And we found that the ASPH expression have relation to the expression of progesterone receptor (PR) and HER-2.

5. Discussion

ASPH is rarely expressed in normal adult tissue, except placental trophoblastic cells [20, 21], however, its over expression has been observed in a number of malignancies. It

plays an important role in tumor development and progression. Wang et al. [21, 22] showed a significant association between ASPH over expression and higher recurrence and lower survival rate after surgery. ASPH has been proposed as an important biological target to control tumor cell migration and invasion, as its over expression has been observed in 70%–90% of human tumors [5, 23]. Breast cancer accounts for 25% of all cancer cases and 15% of deaths in women from female cancers [23]. Age, race, acyeterion use, family history, reproductive and endocrine factors, hormone therapy, obesity, excessive alcohol consumption, smoking, and lack of physical activity have all been identified as potential risk factors for breast cancer [24]. Due to the complex etiology of breast cancer, the prognosis of breast cancer patients has attract more and more attention, especially from the perspective of molecular biomarkers [25]. ER, PR and HER-2 are very important biomarkers in the prognosis of breast cancer patients, and their status is crucial to the prognostic [26]. And they are thought to be predictive markers of breast cancer patients' response to hormone therapy [27]. Our experiments found that ASPH was highly expressed in breast cancer cells (MCF7), and the Immunohistochemistry was positive in 15 patients of 34 breast cancer. At the same time, the relationship between ASPH protein expression and ER, PR and HER2 was also investigated in order to explore whether ASPH could be used as a possible biomarker for the identification of breast cancer.

Our study found that ASPH was over expressed in breast cancer with a positive rate of 44.1%. In addition, our study found that the expression of ASPH in breast cancer tissues was correlated with the expression levels of PR and HER2. Patients with PR and HER2 were highly positive for ASPH expression, i.e. Luminal B (HER2 positive). Luminal type B breast cancer has poor response to endocrine therapy and requires adjuvant chemotherapy with poor prognosis [28, 29]. Masafumi Shimoda etal.[30] showed that patients with luminal B-like breast cancer of high ASPH had a significantly worse prognosis than patients with ASPH-low luminal B-like breast cancer. Our results suggest that ASPH expression synchronizes with HER2, and ASPH may be an important indicator for evaluation breast cancer patients. These results suggest that ASPH may be a potential biomarker for breast cancer diagnosis and prognosis, and lay the foundation for future HER-2 targeted therapy for breast cancer. In addition, we will expand the samples in future studies to examine the clinical value of ASPH in breast cancer. And we will find out the related protein signaling pathways for ASPH.

Declaration of Interest Statement

No interest conflict.

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