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High Expression of Asparagine Synthetase Is Associated with Poor Prognosis of Breast Cancer in Chinese Population

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Abstract

Aims: This study aimed to determine the expression of asparagine synthetase (*ASNS*) in breast cancer (BC) tissues and estimate its prognostic value for BC patients. Besides, the roles of *ASNS* in the proliferation of BC cells were also examined in the study.
Methods: Quantitative real-time PCR was conducted to detect the expression of *ASNS* mRNA in BC tissues and normal controls. The relationship between *ASNS* expression and clinical characteristics of BC patients was analyzed using χ-square test. MTT assay was performed to explore the effect of *ASNS* expression on the proliferation of BC cells. Kaplan–Meier curves were plotted to describe the overall survival rate of BC patients. Cox regression analyses were implemented to investigate prognostic factors.
Results: *ASNS* mRNA overexpression was observed in BC tissues ($p < 0.05$). High expression of *ASNS* was significantly related to histological grade ($p = 0.017$), vascular invasion ($p = 0.009$), and PR status ($p = 0.014$). The downregulation of *ASNS* affected the proliferation of BC cells ($p < 0.05$). Kaplan–Meier survival showed that patients with high *ASNS* expression lived shorter than those with low expressions ($p < 0.001$). Finally, Cox regression analyses revealed that *ASNS* could act as a prognostic marker for BC patients ($p < 0.001$, HR = 3.293, 95% CI = 1.790–6.058).
Conclusion: Taken together, *ASNS* is a valuable prognostic biomarker for BC patients.

Keywords: *ASNS*, breast cancer, prognosis, survival

Introduction

Breast cancer (BC) is one of the most frequent cancers among women of more than 35 years of age in both developing and developed countries.[1,2] The occurrence of BC is associated with heredity, and this disease often attacks women before and after menopause. BC usually derives from glandular epithelium of the breast and severely threatens physical and mental health of the cases. BC is highly heterogeneous at molecular and clinical levels.[3] The characteristics of BC mainly include breast lump, dysgalactia, thoracalgia, and distant metastasis. It has been estimated that about 235,000 women were diagnosed with BC in 2014, and the incidence rate of BC has been increased in recent years.[4,5] Treatments for BC mainly depend on surgical resection and radiotherapy. However, about 5%–45% of cases would face recurrence, producing a great challenge.[6] What is more, although overall survival of BC has been greatly improved in the past decade with the advancements in adjuvant therapy and early detection, this malignancy is still one of the leading causes for cancer-related deaths.[7,8] The 5-year survival rate of BC patients in advanced stage is only 20%.[9] Therefore, it is needed to find novel and efficient ways to better prognosis prediction and treatment of BC.

Asparagine is a nonessential amino acid, and widely required for cellular growth and functions.[10,11] Cells receive asparagine physiologically through two major ways: circulating *ASNS* or plasmatic asparagine.[12] *ASNS* consists of two domains: the N-terminal catalyzing the hydrolysis of glutamine, and the C-terminal catalyzing the carboxylate of aspartate. Asparagine synthetase (*ASNS*) gene encodes ASNS protein, which belongs to the aminotransferase family and catalyzes the 1-glutamine and 1-aspartate into 1-glutamate and 1-asparagine in an ATP-dependent manner.[13–15] Previous studies have claimed that *ASNS* played important roles in cancer cell growth. According to Li et al., the knockdown of

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ASNS could inhibit the growth of melanoma and epidermoid carcinoma cells, indicating it was a candidate target in melanoma treatment.[16] Yang et al. proposed that *ASNS* might contribute to the tumorigenesis of BC, and that decreased expression of *ASNS* inhibited the proliferation of BC cells and induced cell cycle arrest.[17]

In the present study, the authors measured the expression of *ASNS* in BC using quantitative real-time PCR and estimated the role of *ASNS* in the proliferation of BC cells with MTT assay. Furthermore, the prognostic value of *ASNS* in BC patients was also evaluated through Kaplan–Meier method and Cox regression analysis.

Materials and Methods

Patients and specimens

In this study, 135 BC patients who underwent surgical resection in Weihai Municipal Hospital were included. None of them had received any radio/chemotherapy before surgery. Clinical parameters, including age, HER2 status, endocrine therapy, family history, histological grade, vascular invasion, and PR status were collected from electronic record system. Patients who were lost in follow-up were excluded from this study. BC tissue samples were obtained from all recruited participants. In addition, 56 adjacent normal tissue samples were also resected from the patients as controls. This study was supported by the Ethics Committee of Weihai Municipal Hospital. Besides, all participants signed the written informed consents.

Cell lines and cell culture

BC cell line MCF7 was provided by Prof. Moon Hyeong-Gon (Seoul National University, Seoul, Republic of Korea). All cells were cultured in DMEM, which was supplemented with 1 × antibiotic/antimycotic and 10% fetal bovine serum (Gibco) in a humidified atmosphere with 5% CO_2 at 37°C.

siRNA and transient transfection

Human *ASNS* siRNA (si-*ASNS*) and negative control siRNA were obtained from Dharmacon (Chicago, IL). Transient transfection was conducted using Lipofectamine RNAiMax Reagent (Invitrogen) based on corresponding protocols. Then cells were cultured under normal conditions.

MTT assay

MTT assay was performed to detect the proliferation ability of transfected MCF7 cells. First, the transfected cells were incubated in 96-well plates at a density of 2000 cells/well for 0, 12, 24, 36, 48, and 60 h, respectively. Then the cells were coincubated with MTT for 4 h. Finally, 200 μL DMSO was added to each well to terminate reactions. The proliferation ability of MCF7 cells was measured with spectrophotometer at a wavelength of 490 nm.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from all BC tissue specimens and normal controls using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Then the isolated RNA was used to synthesize cDNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Finally, real-time PCR was conducted with the Light-Cycler 480 Real-Time PCR System (Roche Diagnostic GmbH, Manheim, Germany) based on the manufacturer's instructions. GAPDH was used as internal reference. The relative expression of *ASNS* mRNA was analyzed through $2^{-\Delta\Delta CT}$ method. Every sample was measured at least three times.

Statistical analyses

All statistical analyses were carried out in software SPSS 18.0 and Sigmaplot 12.5. All data were presented as mean ± SD. Student's *t*-test was used to determine difference in *ASNS* expression between BC tissues and normal controls. Chi-square test was adopted to analyze the relationship between ASNS expression and clinical features of the patients. Overall survival curves were estimated by Kaplan–Meier method. Finally, the prognostic performance of *ASNS* in BC was evaluated by Cox regression analysis. Two-side *p* value was used in this study, and results were considered to be statistically significant when *p* was less than 0.05.

Results

Upregulated expression of ASNS *mRNA in BC tissues*

The relative expression of *ASNS* mRNA in BC tissues and normal controls was determined by qRT-PCR. The results showed that the relative expression of *ASNS* was significantly higher in BC tissues than in normal controls (Fig. 1, $p < 0.05$).

Relationship between ASNS *expression and clinical features*

Chi-square test was conducted to analyze the association between *ASNS* expression and clinical features of BC patients. According to the median value of *ASNS* expression, the authors divided cancer tissues into high and low expression groups. As shown in Table 1, *ASNS* expression was affected by histological grade ($p = 0.017$), vascular invasion ($p = 0.009$), and PR status ($p = 0.014$). However, there was no significant association for *ASNS* expression with age ($p =$

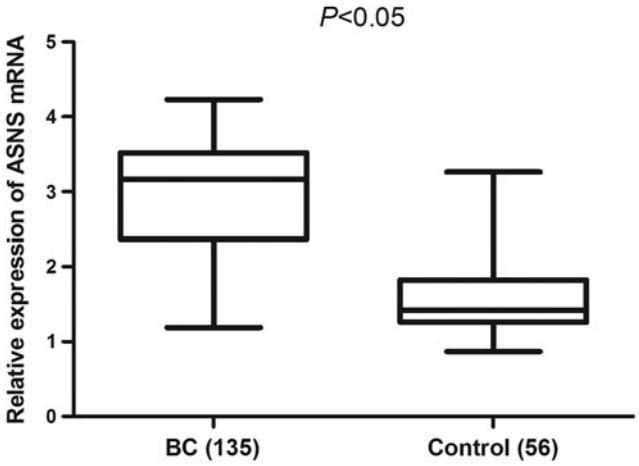

FIG. 1. The expression of *ASNS* mRNA in BC tissues and adjacent normal ones. The result showed that the expression of *ASNS* mRNA in BC tissues was higher than that in normal controls ($p < 0.05$). BC, breast cancer.

Table 1. Relationship Between *ASNS* Expression and Clinical Features of Breast Cancer Patients

Characteristics	*Case No.*	*Expression*		p *value*
		High	*Low*	
Age				0.361
≦45	71	47	24	
>45	64	47	17	
HER2 status				0.222
Positive	70	52	18	
Negative	65	42	23	
Endocrine therapy				0.112
Yes	75	48	27	
No	60	46	14	
Family history				0.191
Yes	74	55	19	
No	61	39	22	
Histological grade				0.017
1,2	78	48	30	
3	57	46	11	
Vascular invasion				0.009
Present	69	55	14	
Absent	66	39	27	
PR status				0.014
Positive	71	56	15	
Negative	64	38	26	

PR, progesterone; HER2, human epidermal growth factor receptor 2.

0.361), HER2 status ($p=0.222$), endocrine therapy ($p=0.112$), or family history ($p=0.191$).

ASNS *knockdown inhibited the proliferation of MCF7 cells*

To explore the role of *ASNS* in the development of MCF7 cells, MTT assay was conducted. All MCF7 cells were transfected by si-*ASNS* and negative control siRNA, and decreased expression of *ASNS* was observed in si-*ASNS* group. As shown in Figure 2, *ASNS* downregulation significantly suppressed the proliferation ability of MCF7 cells ($p<0.05$).

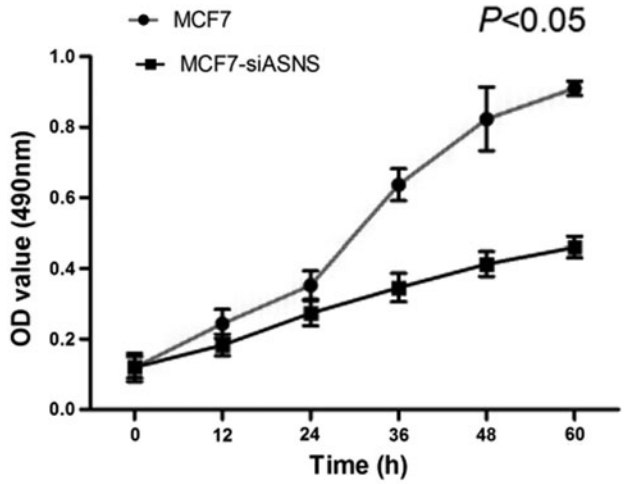

FIG. 2. The effect of *ASNS* on the proliferation of MCF7 cells. The knockdown of *ASNS* inhibited the proliferation of MCF7 cells ($p<0.05$).

The prognostic value of ASNS for BC patients

The Kaplan–Meier method and Cox regression analyses were carried out to determine the prognostic role of *ASNS* in BC patients. All patients were followed up every 3 months in the first 2 years and then every 6 months in the last 3 years. During the follow-up, 79 patients died, including 66 (70.2%) with high *ASNS* expressions and 13 (31.7%) with low expressions. According to Kaplan–Meier curves, patients with high *ASNS* expressions had shorter survival time than those with low expressions (Fig. 3, $p<0.05$). Besides, as listed in Table 2, histological grade ($p=0.003$, HR = 1.954, 95% CI = 1.254–3.046), HER2 status ($p=0.035$, HR = 1.623, 95% CI = 1.035–2.546), PR status ($p=0.001$, HR = 2.194, 95% CI = 1.380–3.487), vascular invasion ($p=0.019$, HR = 1.714, 95% CI = 1.093–2.689), and *ASNS* expression ($p<0.001$, HR = 3.919, 95% CI = 2.151–7.142) were related to the prognosis of BC patients. What is more, multivariate analysis suggested that histological grade ($p=0.019$, HR = 1.709, 95% CI = 1.092–2.674), PR status ($p=0.007$, HR = 1.897, 95% CI = 1.188–3.028), and *ASNS* expression ($p<0.001$, HR = 3.293, 95% CI = 1.790–6.058) were prognostic factors for BC patients.

Discussion

BC is one of the most common invasive malignancies in the world.[18] A variety of biomarkers have been investigated for their roles in the development and progression of BC. Chen et al. revealed that *miR-22* targeted glucose transporter protein type 1 and was a promising marker for the prognosis

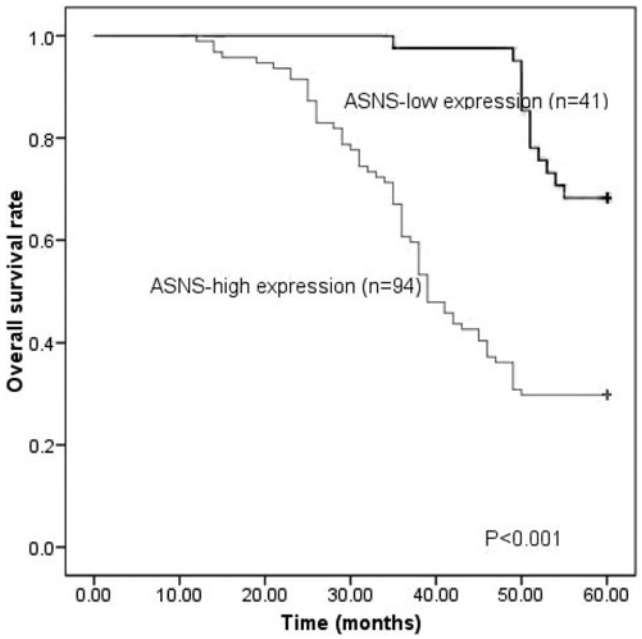

FIG. 3. Survival curves were plotted based on the expression of *ASNS* to evaluate the overall survival rate of BC patients. Patients with high *ASNS* expressions faced higher mortality rate than those with low expressions ($p<0.001$).

Table 2. Univariate and Multivariate Analyses of Clinical Factors

	Univariate		*Multivariate*	
Clinical factors	p *value*	*HR (95% CI)*	p *value*	*HR (95% CI)*
HER2 status	0.035	1.623 (1.035–2.546)	—	—
Family history	0.169	1.373 (0.874–2.158)	—	—
Histological grade	0.003	1.954 (1.254–3.046)	0.019	1.709 (1.092–2.674)
Vascular invasion	0.019	1.714 (1.093–2.689)	—	—
PR status	0.001	2.194 (1.380–3.487)	0.007	1.897 (1.188–3.028)
ASNS expression	<0.001	3.919 (2.151–7.142)	<0.001	3.293 (1.790–6.058)

of BC patients.[19] Sun et al. demonstrated that *miR-200c* enhanced radiosensitivity and suppressed the autophagy of BC cells through regulating *UBQLN1*.[20] In the study of Wu et al., serum levels of CEA and CA15–3 before surgery could act as prognostic biomarkers in BC.[21]This study was the first one to explore the relationship of *ASNS* expression with the prognosis of BC patients.

It is reported that the silencing of *ASNS* in human sarcoma cell lines reduced the percentage of cells at S phase and inhibited the synthesis of new polypeptide.[22] Moreover, there is evidence supporting that *ASNS* is overexpressed in various cancers and related with the development and progression of the cancers, such as in prostate cancer, brain tumors, and lymphoblastic leukemia.[23–25] In the present study, they also assessed the influence of *ASNS* expression on the growth of BC cells. MTT assay showed that cells, after knocking down *ASNS,* proliferated slower than those expressing *ASNS*. The results of this study were consistent with those in previous reports.

In addition, in this study, they first examined the expression of *ASNS* mRNA in BC tissues and healthy controls, and *ASNS* mRNA was positively expressed in BC tissues, which was in accordance with findings in previous reports. The following Chi-square test demonstrated that high *ASNS* expression was related to PR status, histological grade, and vascular invasion. Based on the above results, they speculated that *ASNS* expression might be associated with the occurrence and development of BC. Finally, survival curves suggested that patients with high *ASNS* expressions had poor prognosis compared with those with low expressions. Furthermore, Cox regression analysis highlighted that *ASNS* could be a prognostic biomarker for BC patients. The findings of this study were in accordance with those by Zhang et al., who demonstrated that the expression of *ASNS* was higher in HCC tissues and that patients with lower *ASNS* expression levels had poor prognosis.[26] Therefore, *ASN*S may be a promising therapeutic target for malignant tumors.

Conclusion

In summary, the overexpression of *ASNS* mRNA was observed in BC tissues compared with adjacent normal ones. Chi-square test unveiled that high *ASNS* expression was correlated with certain clinical factors. In addition, the knockdown of *ASNS* by siRNA could significantly inhibit the proliferation of BC cells. Both survival curves and Cox regression analyses proved that *ASNS* was related to the prognosis of BC patients and its upregulation predicted poor prognosis. However, molecular mechanism through which *ASNS* worked in BC has not yet been precisely illustrated, which needs more and further research in the future.

Author Contributions

Conceived and designed the experiments: C.Q.; Performed the experiments: X.Y.; Analyzed the data: Z.Z.; Contributed reagents/materials/analysis tools: C.Q.; Wrote the article: X.Y.

Author Confirmation Statement

All coauthors have reviewed and approved the article before submission.

Disclosure Statement

No competing financial interests exist.

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