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ASPP1/2 positive patients with invasive breast cancers have good prognosis

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A B S T R A C T

Although the expression of ASPP family members in multiple tumors has been studied, especially in various cell lines of breast cancer (BC), but the expressions pattern of ASPP family members in invasive BC tissues are not clear. We studied the expression and expression pattern of ASPPs family member in BCs, the relationship between ASPP family members and clinic-pathologic features of BCs was also analyzed. The results showed that the expression of ASPP1, ASPP2 and iASPP was observed on AE1/AE3^{+} tumor cells, and not on infiltrated lymphocytes and capillaries. The relationship between ASPP1 expression and pTNM stage has statistical difference ($p < 0.01$). The relationship between expression of ASPP2 and SBR grade has statistical difference ($p < 0.05$). The relationship between expression of iASPP and clinic-pathologic feature of patients has no statistical difference ($p > 0.05$). The patients with positive expression of ASPP1 and the patients with negative expression of ASPP1 have statistical difference in 3-year survival rate and 5-year survival rate ($\chi^2 = 4.49$, $P = 0.03$; $\chi^2 = 3.79$, $P = 0.048$). Overall, our work demonstrated that the expression of ASPP1/2 contributes to predict the prognosis of patients with BC.

1. Introduction

Breast cancer (BC) is one of the malignant tumors with high morbidity in female. At present, comprehensive treatment based on surgery is the main clinical treatment to BCs, which has achieved optimal curative effect. Postoperative recurrence, cancer cell metastasis and development of drug resistance are key factors that influence survival time and death rate of patients with BCs [1,2]. Researchers have detected abundant molecular markers for treatment and prognosis of BCs [3,4]. They attempted to distinguish patients with BC of different molecular types, adopt individual and accurate treatment to different molecular types, improve curative effect of BC, and predict survival time of patients [5].

TP53 mutations are independent biomarkers of bad prognosis in patients with BC [6,7], The mortality of BC patients with exon 5–8 mutation of p53 was higher than that of no such mutations [8]. ASPP is the p53 protein downstream regulation molecule which was discovered by Samuels-Lev et al., in 2001 [9]. ASPP genes can regulate cell growth, cell apoptosis and differentiation in various tumors [10,11]. ASPP family has three members, namely, ASPP1, ASPP2 and inhibitory ASPP (iASPP). They have similar molecular structures. They have three typical characteristic structures: 4 ankyrin repetitive sequences, SH3 structural domain and the structural domain with abundant proline. ASPP1/2 can combine with p53 to regulate bioactivity of p53 proteins and promote DNA damaged cells into the apoptosis program. iASPP has different functions, it combines with the p53 protein competitively to inhibit the activity of wild p53

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protein in promoting cell apoptosis. Therefore, iASPP has characteristics of cancer genes. ASPP family expressed in multiple tumor tissues [12–14]. We have explored expressions of ASPP family in various cell lines of BC preliminarily and found that ASPP family has different expressions in them [15]. Our previous study showed that ASSP1 and ASSP2 induced cell apoptosis by activation of p53, whereas iASPP inhibited the function of p53 protein. In this paper, expressions pattern of ASPP family members in invasive BC tissues were studied, aiming to further understand expressions of ASPP family members in BC as well as their clinical significances and biological functions. Moreover, the possibility of using ASPP family members as potential molecular targets for treatment was also explored.

2. Results

General data. A total of 155 female patients with BC were collected. They aged from 25 to 84, showing an average age of 60 ± 14.7 years. The follow-up time lasted for 6–108 months and the survival time was 2~108^{+} months, with an average survival time of 65.17 ± 21.99 months. No radiotherapy and chemotherapy have been given to patients before the surgical treatment. And the SBR grade of

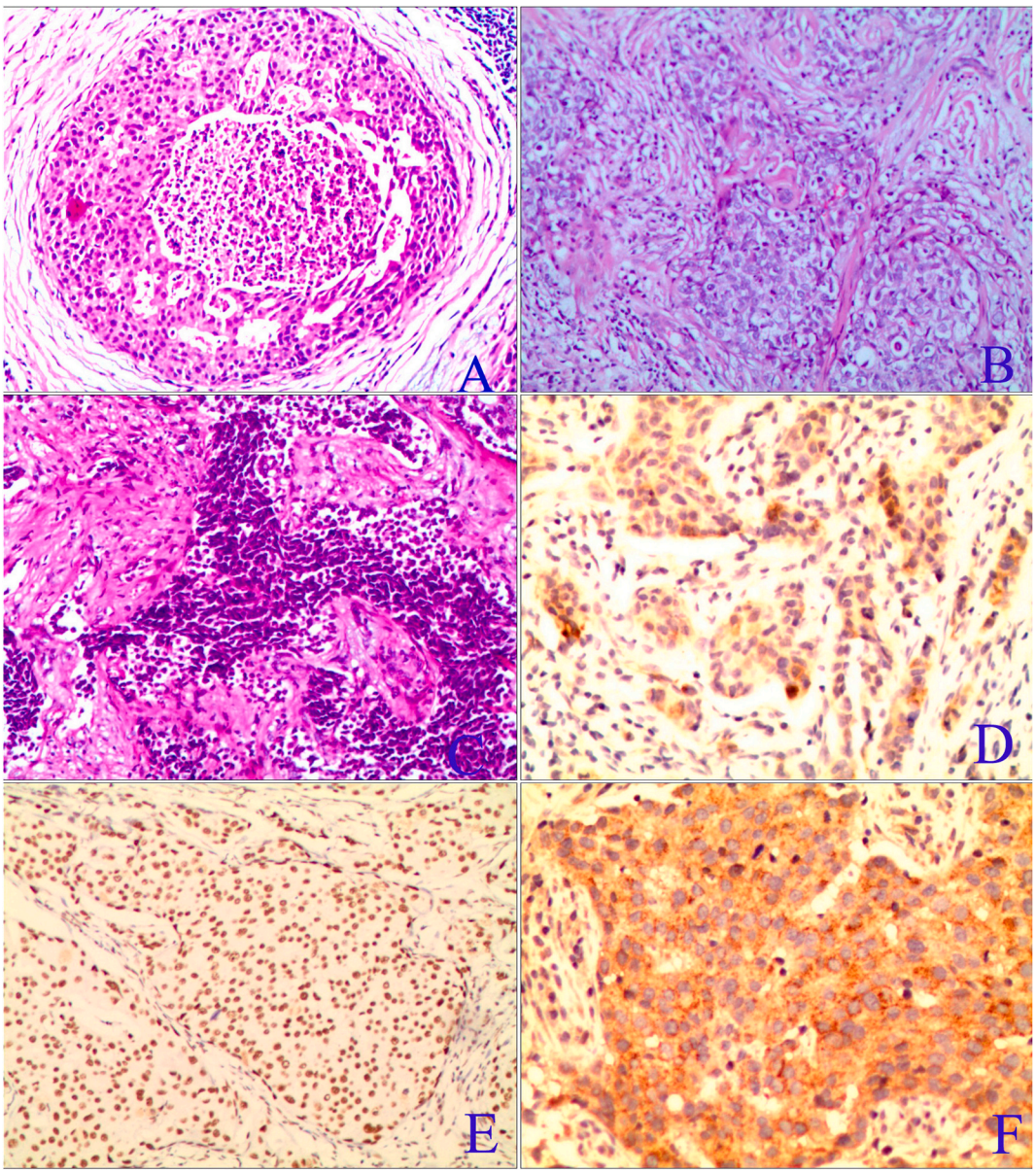

Fig. 1. SBR grade of breast cancer and expression of ASPP family members in breast cancer. SBR gradeI(A), SBR gradeII(B), SBR gradeIII(C), (hematoxylin-eosin staining, Magnification × 100). The tumor cells were positive for ASPP1 (D, Magnification × 200), ASPP2 (E, Magnification × 100) and iASPP (F, Magnification × 200).

breast cancer tissue was shown in Fig. 1 (grade I,1A; gradeII, 1B; grade III, 1C).

Expressions of ASPP family members in BC tissue. Observation of brown particles in cancer cell cytoplasm, nucleus and/or on membrane are deemed positive expression. Results showed that: in 155 patients with breast cancer, the positive expression rate of ASPP1 is 13.55 % (21/155) (Fig. 1D), positive expression rate of ASPP2 is 97.42 % (151/155) (Fig. 1E), and the positive expression rate of iASPP is 61.29 % (95/155) (Fig. 1F).

Expression pattern of ASPP family members. To display the anatomical pattern of ASPP family members in BC cancer better, the fluorescence dual staining was further examined. The results showed that the expression of ASPP1, ASPP2 and iASPP was observed on AE1/AE3^{+} tumor cells (Fig. 2). AE1/AE3 was a special antibody which was expressed in the cytoplasm of epithelial cell. Furthermore, the CD45 (LCA) antibody was also used in this study to verify the expression of ASPP family members were on tumor cells instead of infiltrated lymphocytes and/or capillaries, interestingly ASPP family members were found to be expressed on epithelial cells and not infiltrated lymphocytes (Fig. 3). At the same time, tumor-associated ASPP1 was found to be co-expressed with ASPP2 and iASPP (Fig. 4). Our results showed that ASPP1, ASPP2 and iASPP are expressed on epithelial breast tumor cells.

Relationship between ASPP family members and clinic-pathologic features of breast cancer. Relationships between ASPP family members and age of patients with BC, maximum tumor diameter, pathological grade, lymphatic metastasis and pTNM stage as well as statistical analysis results are shown in Table 1. The relationship between ASPP1 expression and pTNM stage has statistical inter-group difference ($p < 0.01$). The relationship between expression of ASPP2 and SBR grade has statistical inter-group difference ($p < 0.05$). The expression of ASPP2 in patients with breast cancer SBR grade II was 51.68 %, while in patients with SBR grade III patients was 17.45 %. The relationship between expression of iASPP and clinic-pathologic feature of patients has no statistical inter-group difference ($p > 0.05$) (Table 1).

Relationship between expressions of ASPP family members in BC and survival time. According to statistical analysis, the 3-year survival rate of study objects is 84.52 % (131/155) and the 5-year survival rate of is 79.35 % (123/155). The patients with positive expression of ASPP1 and the patients with negative expression of ASPP1 have statistical difference in 3-year survival rate and 5-year survival rate (Fig. 5, Table 2). This prompts that ASPP1 could be used to predict short and long term survival time of patients with

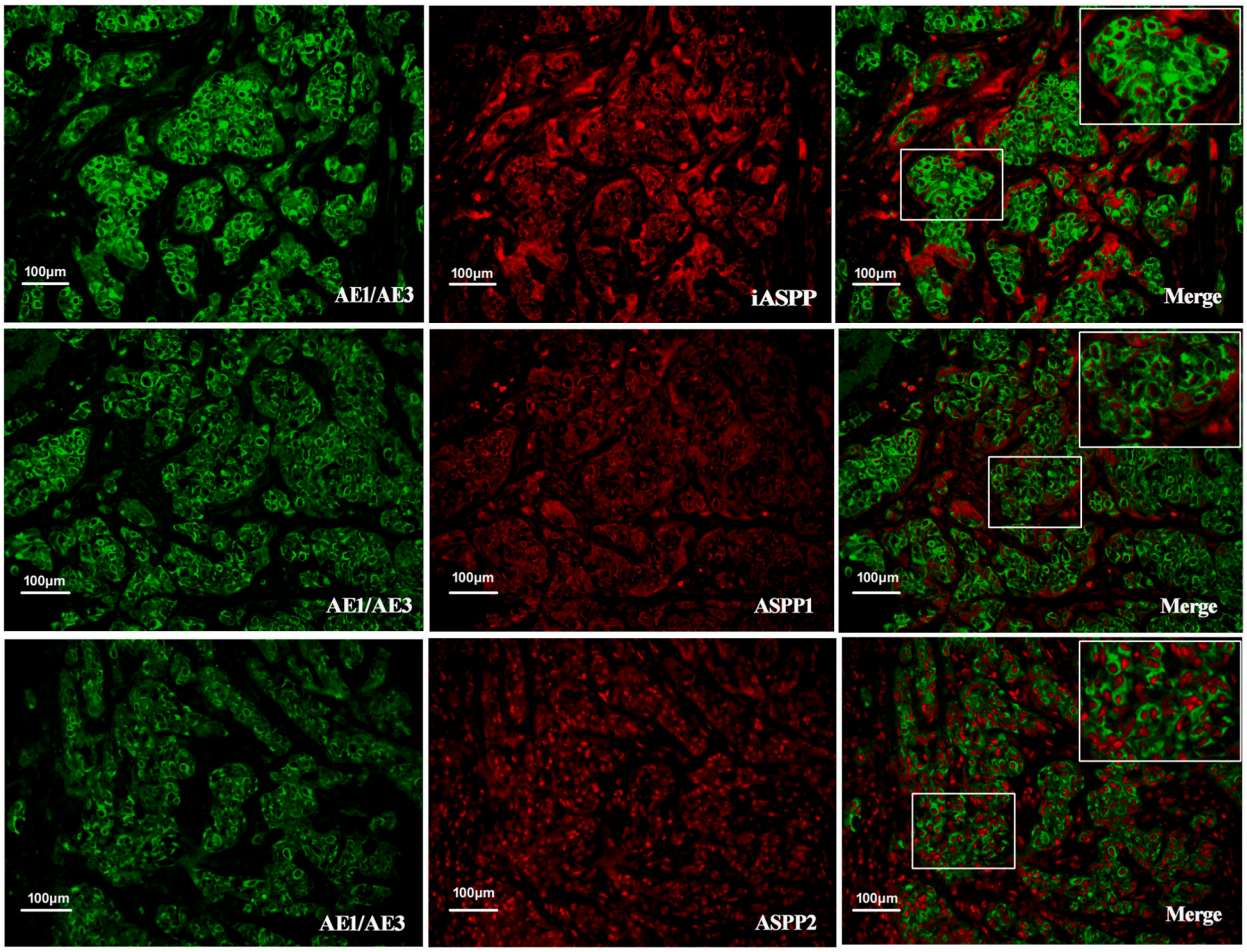

Fig. 2. The relationship of ASPP family members and AE1/AE3^{+} tumor cells in BC. The expression of ASPP family members and AE1/AE3 in breast cancer was detected by fluorescence dual staining. The results showed that ASPP1, ASPP2 and iASPP were found on AE1/AE3^{+} tumor cells.

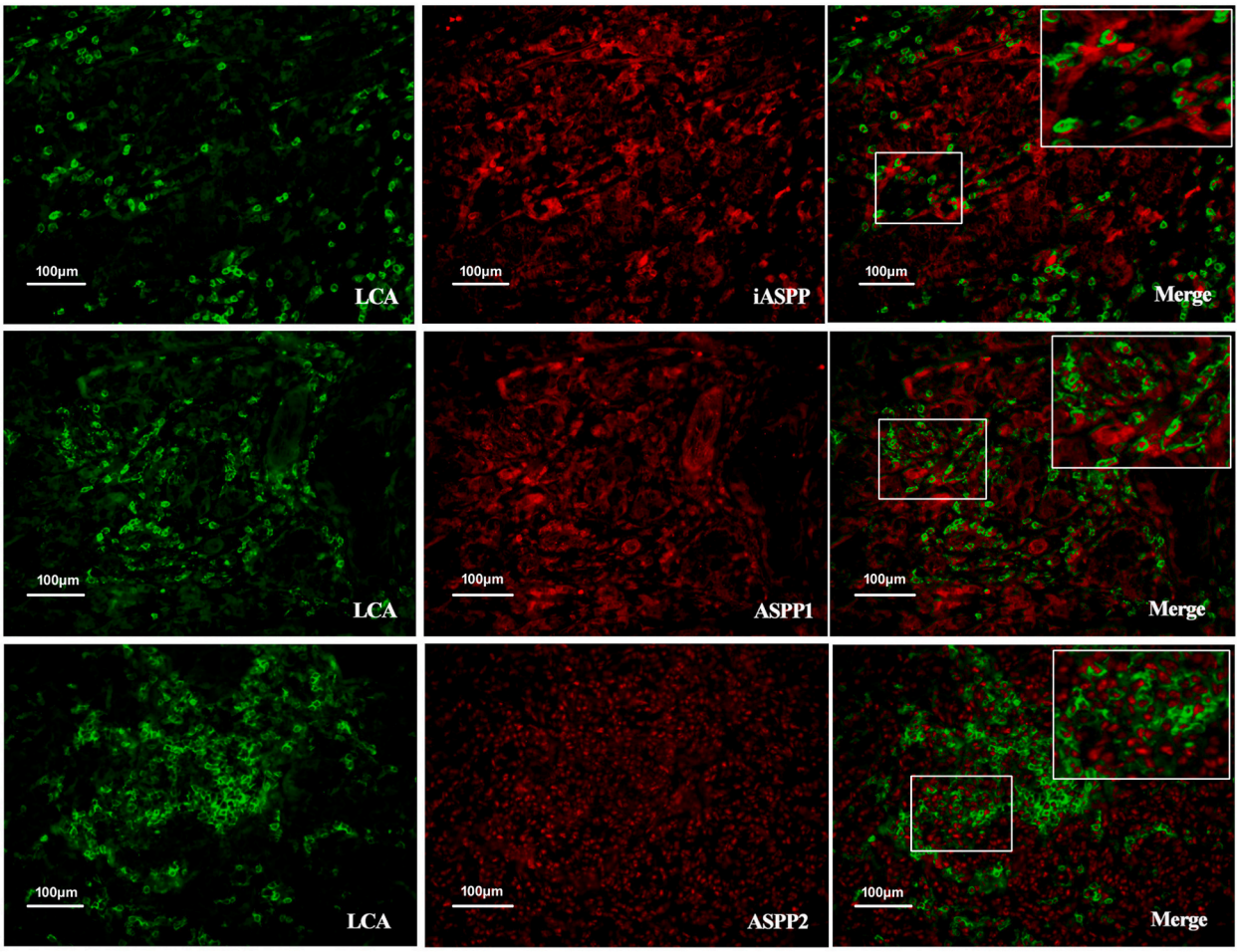

Fig. 3. The relationship of ASPP family members and CD45^{+} inter-tumor infiltrated lymphocytes in breast cancer. The expression of ASPP family members and CD45 in breast cancer was detected by fluorescence dual staining. The results showed that ASPP1, ASPP2 and iASPP were found on tumor cells, and not on CD45^{+} infiltrated lymphocytes and monocytes.

invasive BC.

The 3-year survival rate of the patients with negative expression of ASPP2 decreases to about 70 % and then keeps stable. The patients with positive expression of ASPP2 have about 90 % of 3-year survival rate and about 70 % of 5-year survival rate. However, statistical analysis finds no statistical difference between the patients with negative expression of ASPP2 and the patients with positive expression of ASPP2 in term of 3-year and 5-year survival rates. This may be related with the low negative expression rate of ASPP2 and abnormal distribution of data. The patients with positive and negative expressions of iASPP have no statistical difference in 3-year and 5-year survival rate, too (Fig. 5, Table 2).

3. Discussion

BC is one of malignant tumors with multiple gene participation. How to increase curative effect and survival time of patients with breast cancer? Predicting survival time of patients is an important clinical topic nowadays. With the development of molecular medicine, the gene expression pattern of breast cancer was analyzed from molecular level by combining the traditional BC histopathology type and gene immunophenotyping, which can increase accuracy of effective therapeutic target screening and judgment prognosis of BC. Results can provide effective references to guide clinical treatment, increase therapeutic effect and prolong survival time of patients.

Researchers have reported differential expressions of ASPP family members in multiple tumors, such as breast cancer [15], neoplastic hematologic disorder [16], non-small cell lung cancer [17], cervical cancer [18], colorectal cancer [14], liver cancer [19] and head-neck carcinoma. This is mainly manifested by low expression of ASPP1/2 and high expression of iASPP. And reduced ASPP1 and ASPP2 expression was associated with advanced disease stage and moderate differentiation in oral cancers progression [20]. However, an increased expression of ASPP2 in patients with neuroblastoma may be associated with poor survival [21]. Even the ASPP

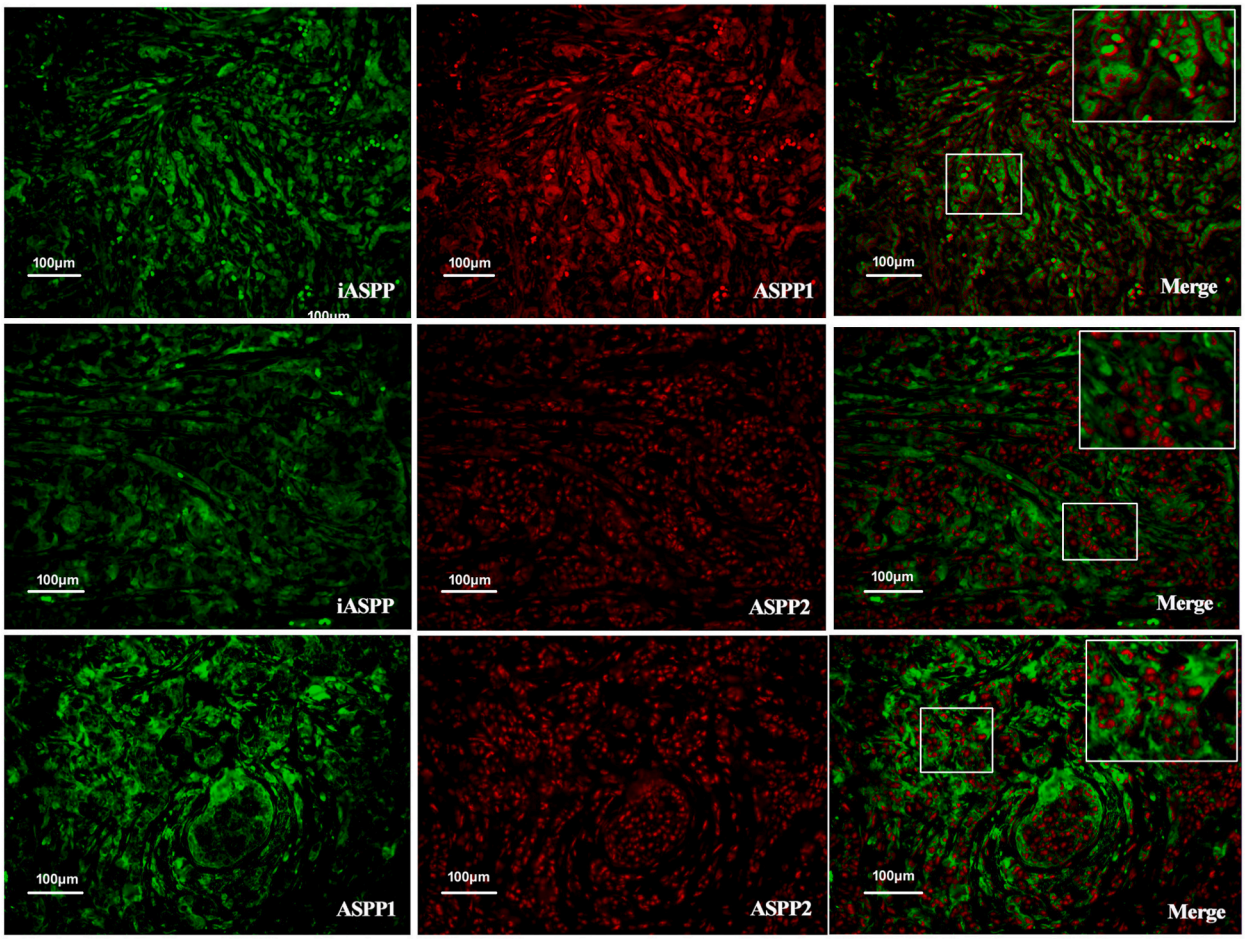

Fig. 4. The expression patterns among ASPP family members in BC. The results showed that ASPP1, ASPP2 and iASPP were found on epithelial tumor cells (fluorescence dual staining).

Table 1
The relationship between ASPP family members and clinic-pathological characteristics of breast cancer.

clinic-pathological	ASPP1		ASPP2		iASPP	
characteristics	Number	*p* value	Number	*p* value	Number	*p* value
Age (year)						
≤50	12	0.770	81	0.661	53	0.599
> 50	9		70		42	
Tumor size (cm)						
≤2	7		38		23	
2-5	10	0.742	79	0.789	55	0.123
≥5	4		34		17	
SBR grade						
I	8		46		29	
II	10	0.705	78	0.014	50	0.825
III	3		27		16	
Lymph nodes metastasis						
0	14		86		53	
1-3	6	0.218	33	0.796	24	0.171
4-9	0		23		14	
≥5	1		9		4	
pTNM stage						
I	5		33		19	
II	14	0.006	83	0.541	57	0.246
III+IV	2		35		19	

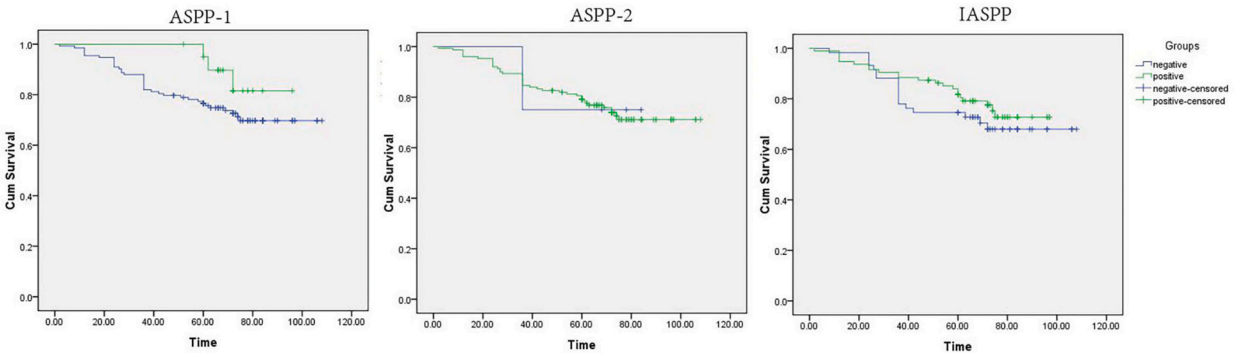

Fig. 5. Kaplan–Meier survival plots presenting patients with BC of ASPP family members expression.

Table 2
Statistical analyze results of survival rate between ASPP family members positive and negative expression in patients with breast cancer.

	3-year survival rate		5-year survival rate	
	Pearson Chi-Square	*P* value	Pearson Chi-Square	*P* value
ASPP1	4.49	0.03	3.79	0.048
ASPP2	0.28	0.60	0.04	0.83
iASPP	3.02	0.08	1.25	0.26

may be potential biomarkers in the treatment of cervical cancer [22] and choriocarcinoma [23]. iASPP also plays a critical role in cytokinetic abscission, the last step of cell division [24].

In our early study, differential mRNA expressions of ASPP family members in various BC cell strains have been discovered by RT-PCR method [15,25]. iASPP mRNA has high expressions in MCF-7 cells, while ASPP1 and ASPP2 have low expressions. Apoptosis of BC cells can be promoted by inhibiting expressions of iASPP [26]. ASPP family members have differential expressions in vitro. How their expressions in BC tissues?

Experimental results in this paper demonstrated that ASPP2 has the highest expression rate in breast cancer (97.42 %, 151/155), followed by the positive expression rate of iASPP (61.29 %, 95/155). ASPP1 has the lowest expression rate (13.55 %, 21/155). Such differential expressions have significantly different expressions of ASPP family members in *in-vitro* cell strains. There's over-expression of ASPP2 in BC tissues, indicating that ASPP2 plays an important role in biological effect of breast cancer. It is reported that expression of ASPP1 in human tumors is down regulated and functions of ASPP1 and ASPP2 have no indistinct substitution [13].

In this experiment, the relationship between expression of ASPP1 and pTNM stage in the BC has inter-group difference. ASPP1 has high positive expression in stage I + II, but low expression rates in stage III + IV, indicating the BC patients with positive expressions of ASPP1 are early stage. How about the relationship between the expression of ASPPs and SBR grade, a prominent prognostic marker in breast cancer? The results displayed the expression of ASPP2 and SBR grade has inter-group difference. With the increase of SBR grades, the positive expression level of ASPP2 increases gradually. In other words, tumor differentiation reduces gradually and tumor malignancy intensifies with the increase of histological grade, resulting in continuous increase of ASPP2 expressions. In the SBR III, expression of ASPP2 disappears gradually in the worsening process of tumor differentiation. A research displayed that ASPP2 is an independent risk factor in hepatocytes carcinoma [27]. Although iASPP has high expression rate in BC tissues, it has no statistical difference with clinic-pathologic feature, which may be related with tissues. But the relationship between expressions of iASPP and lymphatic metastasis has statistical inter-group difference in colorectal cancer according to our discoveries [28]. The loss of ASPP1 enhanced the migration and invasion of colorectal carcinoma in vivo and in vitro, the results were obtained through the activation of Snail-2 and then the epithelial-mesenchymal transition was induced. The authors presumed that the ASPP1 was a prognostic marker for colorectal carcinoma [29].

Survival time of patients with BC is influenced by many factors, such as age of onset, tumor size, histological type, pathological stage, molecular subtype, clinical stages, etc. However, these influencing factors are not independent prognostic factors. In this paper, survival time of patients with BC was analyzed by expressions of ASPP family members. Prognosis of patients with BC was predicted by combining expressions of ASPP family members and survival time of the patients. Results showed that at the end of follow-up time, 40 patients died (25.81 %) and 115 patients survived. The shortest survival time is 2 months. According to statistical analysis, the patients with positive and negative expressions of ASPP1 have statistical difference in 3-year and 5-year survival rates, indicating that ASPP1 can be used to predict short and long survival time of patients with invasive BC. The 3-year survival rate of patients with positive expression of ASPP2 is about 90 % and the 5-year survival rate decreases to about 70 %. The 3-year survival rate of patients with negative expression of ASPP2 is about 70 %. Nevertheless, patients with positive and negative expressions of ASPP2 have no statistical difference in 3-year and 5-year survival rate. It is speculated that this may be related with over-expression of ASPP2 in BC and abnormal distribution of data. Patients with positive and negative expressions of iASPP have no statistical difference in 3-year and 5-

year survival rate. Lossos et al. [30] discovered that among patients with diffuse large B-cell lymphoma and follicular lymphoma, the patients with high expression of ASPP2 have longer survival time, which reflects that expression of ASPP2 may be related with prognosis of malignant tumor. The patients with high expression of iASPP have strong invasion of tumor cells. High expression of iASPP can promote tumor growth and is easy to cause cancer cell metastasis, resulting in poor prognosis.

In a word, expression patterns of ASPP family members in invasive BC tissues and cell strains are different markedly. Expressions of ASPP1/2 are related with pTNM stage, histological grade and prognosis of breast cancer. ASPP family members can be used as important indexes to judge clinical stage, progress and prognosis prediction of BC.

4. Materials and methods

4.1. Sample selection

A total of 155 cases with invasive BC who have received operative treatment from January 2005 to April 2010 in the 989th hospital were collected as the study objects. All patients have excision, definite pathological diagnosis, complete clinical data and detailed follow-up data, including age, gender, type of pathologic histology, tumor volume, pathological stage, lymphatic metastasis, pathologic histological grade, distant metastasis and survival time. The patients with incomplete data or loss of follow-up were deleted. This study complied with the Declaration of Helsinki and approved by the Review Board of the 989th Hospital, PLA. Written informed consent was obtained from the family members of the patient for publication of this article and any accompanying images.

4.2. Immunohistochemical Staining

The 10 % neutral buffered formalin fixed and paraffin-embedded tissues samples were sliced in 2-μm-thick sections, the sections were hematoxylin and eosin stained and immunohistochemical detected. For immunohistochemical staining, sections were mounted on poly-lysine treated glass slides. Endogenous peroxidase activity was blocked with 3.0 % H_2O_2 for 15 min. The sections were pressure cooked for 15 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval after being dewaxed with xylene and rehydrated through a graded series of ethanol. Sections were then incubated at 4 °C overnight with primary antibody. Rabbit anti-human ASPP1 multiclonal antibody (ab51831), rabbit anti-human ASPP2 monoclonal antibody (EPR13837) and rabbit anti-human iASPP multi-clonal antibody (ab115605) were from Abcam. The work solutions were ASPP1 (1:300), ASPP2 (1:1000), iASPP (1:800) respectively. On the next day, sections were washed and incubated with the secondary antibodies for 1 h at RT. Peroxidase activity was visualized with DAB (DAKO), and then the sections were lightly counterstained with hematoxylin. Sections incubated without primary antibody as negative control were done at the same time.

4.3. Immunohistochemical results assessment

ASPP1 was observed in the cytoplasm and nuclei of tumor cells, ASPP2 was found in the nuclei, whereas iASPP was found in the cell cytoplasm. The brown particles were observed in the cytoplasm and/or nuclei under the microscope to presume positive expression.

4.4. Dual immunofluorescence staining

The formalin-fixed and paraffin-embedded tumor tissue were sliced in 3 μm-thick sections, and then the dewaxed and rehydrated sections were incubated with primary antibodies, mouse anti-human ASPP1 monoclonal antibody (1:300), rabbit anti-human ASPP2 monoclonal antibody (1:1000), rabbit anti-human iASPP multi-clonal antibody (1:800), mouse anti-human Cytokeratin (AE1/AE3) monoclonal antibody and mouse anti-human CD45 (LCA) monoclonal antibody at 4 °C overnight. After being washed with 0.1 % PBS, Alexa Fluor 594-conjugated goat anti-mouse/rat/rabbit IgG antibodies, Alexa Fluor488-conjugated goat anti-mouse/rat/rabbit IgG antibodies or fluorescein isothiocyanate (FITC)-conjugated mouse anti-goat IgG antibodies (Beyotime Biotechnology, Shanghai, China) were added and incubated for 1 h. At the same time, the appropriate isotype control primary antibodies and fluorescently labeled secondary antibodies were used as isotype controls. The final results were observed by using fluorescence microscopy (Zeiss Axioplan, Jena, Germany).

4.5. Statistical analysis

Relationship between ASPP family members and clinic-pathologic features of BCs was analyzed by Pearson chi-square test, and survival analysis was done by Kaplan-Meier analysis. All analyses were done using the SPSS software17.0. A difference was considered statistically significant if $p < 0.01$ or $p < 0.05$.

Ethical statement

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the 989th Hospital of the PLA Joint Logistic Support Force review board (No. 20190028).

Data availability statement

The data generated or analyzed during this study are included within this article and available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Changsong Wang: Conceptualization, Writing – review & editing. **Ke Li:** Conceptualization. **Junling An:** Conceptualization, Validation, Writing - review & editing. **Xuexia Lv:** Formal analysis, Writing – review & editing. **Wenfeng Ma:** Data curation, Investigation, Methodology. **Nianlong Meng:** Data curation, Formal analysis, Methodology. **Tian Yun:** Formal analysis, Methodology. **Ting Zhao:** Data curation, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20613.

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