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Inhibitory member of the apoptosis-stimulating protein of p53 is overexpressed in bladder cancer and correlated to its progression

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Abstract

Several lines of direct evidence show that inhibitory member of the apoptosis-stimulating protein of p53 (iASPP) has an important function in cancer progression. However, its expression pattern and relationship with clinical pathologic characteristics in bladder cancer (BC) have not been completely elucidated. In this study, firstly, samples from 3 patients with invasive BC were detected by liquid chromatography tandem mass spectrometry to confirm overexpression of iASPP in BC, then samples from patients with noninvasive and invasive BC were detected by real-time polymerase chain reaction, Western blot, and tissue microarry immunohistochemistry. The relationship between iASPP expression and various clinicopathological features was investigated. The results showed m-RNA and protein of iASPP were overexpressed in BC and the rate of iASPP-positive cells was positively correlated with Union for International Cancer Control-Tumor, Node, Metastases stage, histologic grade, lymph node metastasis and poor overall survive. The data demonstrate that iASPP is overexpressed in BC and promotes the malignancy of BC. iASPP maybe serve as a potential therapeutic target for BC.

Abbreviations: ASPP = apoptosis-stimulating protein of p53, BC = bladder cancer, iASPP = inhibitory member of the apoptosis-stimulating protein of p53, LC-MS = liquid chromatography tandem mass spectrometry, OS = overall survival, SPSS 21.0 = statistics package for social science 21.0, TMA-IHC = tissue microarry immunohistochemistry.

Keywords: bladder cancer, iASPP, malignancy

1. Introduction

The incidence and mortality of bladder cancer (BC) is the second in the genitourinary system cancer[1] and it is the 9th most common malignancy worldwide,[2] accounting for an estimated 386,000 new diagnoses and 150,000 deaths each year.[3]

The apoptosis-stimulating protein of p53 (ASPP) family consisting of 3 proteins (ASPP1, ASPP2, and inhibitory member of the apoptosis-stimulating protein of p53 "iASPP") interacts with and modulates the functions of wild-type (WT) p53.[4] ASPP1 and ASPP2 bind to p53 and aid transcription of proapoptotic genes,[5] whereas iASPP inhibits the apoptotic transactivation potential of p53 by direct interaction.[6] Therefore iASPP was considered as an oncogene. iASPP also regulates cell proliferation[7] and apoptosis.[8,9] Although mutation of the p53 was frequent in a wide variety of human cancer, iASPP plays an important role in human cancers, such as breast cancer[8] and acute leukemia.[10] Elevated expression of iASPP correlates with poor survival in head and neck cancer,[11] oral squamous cell carcinoma,[12] cervical cancer,[13] and ovarian cancer.[14] Increased iASPP expression is associated with tumor grade, invasion, and lymph node metastasis in endometrial cancer.[15]

However, little is known about the role of iASPP in BC. In this study, we explored the expression patterns of iASPP and relationship with clinical pathologic characteristics in BC. Our data demonstrate that iASPP is overexpressed in BC and promotes the malignancy of BC. iASPP may serve as a potential therapeutic target for BC.

2. Materials and methods

2.1. Patients and samples

Cancer and paracancer samples from 144 primary bladder cancers at our institution from 2008 to 2010 were collected at cystectomy or transurethral resection of bladder tumor and snap-frozen in liquid nitrogen. Use of tissue for this study was approved by the Institutional Review Board of Nanjing Medical University (IRB00001934). Tumors were graded histologically in accordance with the World Health Organization classification and were staged as per the Tumor, Node, Metastases staging system of the Union for International Cancer Control. All patients provided written informed consent. All patients have not received chemotherapy in the preoperation. Forty-five patients

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Table 1

iASPP expression in relation to clinicopathological characteristics of BC.

Variables	Case (n)	iASPP expression		P
		Negative (%)	Positive (%)	
Age, y*				.607
<70	81	21 (25.9)	60 (74.1)	
≥70	63	14 (22.2)	49 (77.8)	
Sex				.820
Female	39	10 (25.6)	29 (74.4)	
Male	105	25 (23.8)	80 (76.2)	
Tumor stage†				<.001
pTa∼1	46	26 (56.5)	20 (43.5)	
pT2∼4	98	9 (9.2)	89 (90.8)	
Histological grade				<.001
Low	51	32 (62.7)	19 (37.3)	
High	93	3 (3.2)	90 (96.8)	
lymph node metastasis				<.01
Negative	89	30 (33.7)	59 (66.3)	
Positive	55	5 (9.1)	50 (90.9)	

BC = bladder cancer, iASPP = inhibitory member of the apoptosis-stimulating protein of p53
* Median, 67 years and range, 33–92 years.
† Staging and grading according to the World Health Organization 2004 classification system.

received cystectomy and 99 patients received transurethral resection of bladder tumor. Patients who received transurethral resection of bladder tumor received intravesical chemotherapy in the postoperation. The clinical demographic details of the 144 patients are outlined in Table 1.

2.2. Analysis of proteomic profile in tissues by Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Q Exactive UPLC-MS/MS)

Three high-grade invasive BC and paired paracancerous tissues were initially selected to explore their different proteomic profile by CapitalBio Corporation (Beijing, China) using ultra-performance liquid chromatography coupled with Q Exactive ultra performance liquid chromatography with mass sepectrometry (UPLC-MS)/MS (Thermo Fisher Scientific, San Jose, CA) according to previous studies.[16–18]

Briefly, tissues were lysed and combined with sodium dodecyl sulfate (SDS) lysis buffer (4% w/v SDS, 100 mmol/L Tris-HCl, pH 7.6), and then incubated at 95°C for 5 minutes, briefly sonicated, and centrifuged at 14,000*g* for 15 minutes. The protein concentration of lysate was determined by bicinchoninic acid assay (Thermo Fisher Scientific). Finally, the lysate was labeled with TMT Mass Tagging Kits (Thermo Fisher Scientific), and loaded to Q Exactive UPLC-MS/MS for detection. The data were analyzed by Proteome Discoverer software (Thermo Fisher Scientific).

2.3. Real-time polymerase chain reaction

The total RNA of cancer and paracancer samples from 10 invasive BC was analyzed using protocol described previously by Li.[19] The total RNA was isolated using UNIQ-10 Spin Column RNA Purified Kit (Sangon, Shanghai, China). The first strand cDNA was synthesized using RevertAidTM First Strand cDNA Synthesized Kit (Fermentas, Burlington, Canada). First Strand cDNA was subsequently subjected to Corbett RG-6000 polymerase chain reaction (PCR) system (QIAGEN, Dusseldorf, German) using FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland). The reactions were optimized by varying the annealing temperatures from 50°C∼55°C; the sense and antisense primers were synthesized as follows: GAPDH 5′-GCAAGTTCAACGGCACAG-3′, 5′-GCCAGTAGACTCCAC-GACAT-3′; iASPP 5′-GGCGGTGAAGGAGATG-3′, 5′- TGAT-GAGGAAATCCACGATAGAG-3′.

2.4. Western blot

Western blot was performed for cancer and paracancer samples from 18 invasive BC using protocol according to the previous studies.[20,21] Aliquots of total protein (50 μg per lane) were electrophoresed on a 12% SDS-polyacrylamide gradient gel and transferred to nitrocellulose membranes (Millipore). Washed in rinse buffer at room temperature and incubated in blocking buffer (5% fat-free milk in rinse buffer) for 30 minutes, the membranes were incubated for 2 hours at room temperature with iASPP (1:100) (Atlas Antibodies, Sigma-Aldrich, UK). Further washed with rinse buffer, the membranes were incubated with 1:1000 diluted horseradish peroxidase-conjugated secondary antibody (Santa Cruz) for 2 hours at room temperature, followed by developing with enhanced chemiluminescence reagents (Amershame, Little Chalfont Buckinghamshire, UK). In addition, β-actin was used as a reference protein. The optical densities were analyzed by using ImageMasterTM2D Platinum (Version 5.0, Amersham Biosciences, Piscataway, NJ).

2.5. Tissue microarry Immunohistochemistry

Immunohistochemistry (IHC) was performed using protocol described previously by Gurung.[22] In brief, the tissue microarray (TMA) slides were deparaffinized, rehydrated, and washed and endogenous peroxidase was blocked using Bond-III "Dewax Protocol D" following the manufacturer's instructions (Leica Biosystems, Newcastle, UK). Epitope retrieval was achieved using Bond-III "Protocol H1(30)" (Leica). The slides were incubated with antibodies against iASPP (1:100) (Atlas Antibodies, Sigma-Aldrich, UK) at room temperature for 1 hour. Antibody binding was detected using diaminobenzidine with hemotoxylin counterstaining following Bond-max and Bond-x "IHC protocol F" (Leica). External controls were noncancer colon tissue (positive) and noncancer liver tissue (negative).

The score of immunohistochemistry was assessed using the methods according to the previous study.[22] The cores were examined under a light microscope at 400× magnification, standardizing the scoring according to the reference control cores. The reference material was assigned a staining intensity of 2, graded on a scale of 0 to 3, against which the bladder cancer cores were compared. Three independent investigators, blinded to the clinical data, assessed the cores with the primary investigator scoring the staining a second time to assess intraobserver variance. At least 5 areas of each core were viewed and the proportion of cells in each core staining positively was assigned a score (0∼75%). A semiquantitative histopathology score was obtained by multiplying the staining intensity score with the percentage score (0∼300). A histopathology score higher than the median was considered positive.

2.6. Statistical analysis

Statistical analysis was performed using statistics package for social science 21.0 (SPSS 21.0; SPSS Inc, Chicago, IL). The

expression of iASPP mRNA and protein of cancer and paracancer samples was analyzed using independent *t* test. Associations between iASPP expression and the clinicopathological characteristics were analyzed using Person χ^2 or Fisher exact test. Five-year overall survival (OS) was the primary outcome measure, estimated using the Kaplan-Meier method and differences in survival among groups were compared using the log-rank test. $P < .05$ were considered statistically significant.

3. Results

3.1. Overexpression of iASPP in BC identified by liquid chromatography tandem mass spectrometry

In the initial study, 3575 proteins were identified in 3 paired BC and the matching paracancerous tissues by Q Exactive UPLC-MS/MS. Protein upregulated or downregulated at least 1.5 times between cancer and paracancer samples as defined screening criteria. Finally, the screening results showed 165 proteins were 1.5-fold differentially expressed ($P < .05$) in which 146 proteins were downregulated and 19 proteins were upregulated, with a permutation-based false discovery rate < 0.05 (Supplemental Table S1, http://links.lww.com/MD/B650). Among the differentially expressed proteins, the expression of iASPP was 1.645-fold higher in BC than that in paracancerous tissues ($P = .005$) (Supplemental Table S1, http://links.lww.com/MD/B650).

Because a limited number of pooled samples were analyzed, findings based on statistics would not be reliable. Therefore, more samples were detected by real-time PCR (RT-PCR) and Western blot to confirm whether overexpression of iASPP in BC.

3.2. iASPP mRNA expression detected by RT-PCR

The iASPP mRNA of cancer and paracancer samples from 10 invasive BC was detected by RT-PCR. iASPP gene level in cancer samples was ~11.1-folds higher than that in paracancer samples, the difference between the cancer and paracancer samples was statistically significant (Fig. 1A).

3.3. iASPP protein expression detected by Western blot

iASPP protein of cancer and paracancer samples from 10 invasive BC was detected by Western Blot. Semiquantitative assay showed iASPP protein level in cancer samples was higher than that in paracancer samples; the difference between the cancer and paracancer samples was statistically significant (Fig. 1B).

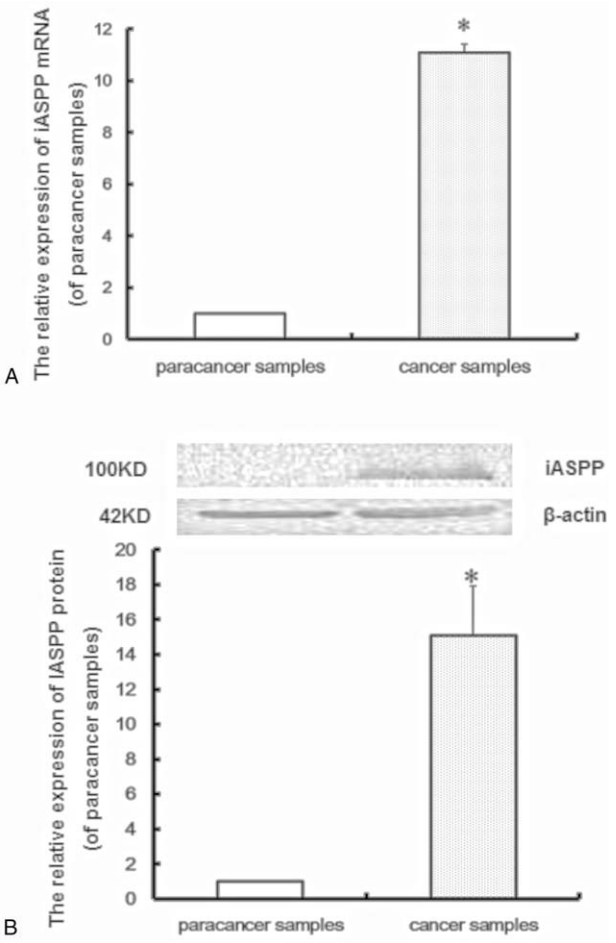

Figure 1. iASPP mRNA and protein expression were detected by real-time RT-PCR and Western blot. (A) The iASPP mRNA of cancer and paracancer samples from 10 invasive BCs was detected by RT-PCR. iASPP gene level in cancer samples was ~11.1-fold higher than that in paracancer samples. (B) The iASPP protein of cancer and paracancer samples from 10 invasive BC was detected by Western Blot. Semiquantitative assay showed iASPP protein level in cancer samples was higher than that in paracancer samples. *vs. paracancer samples, $P < .05$. BC = bladder cancer, iASPP = inhibitory member of the apoptosis-stimulating protein of p53, RT-PCR = real-time polymerase chain reaction.

3.4. TMA-IHC assessment of iASPP expression

iASPP signal intensity was graded zero, weak, moderate, and strong. Strong cytosolic staining was found in cancer samples from patients with invasive BC (Fig. 2C), moderate cytosolic staining was found in cancer samples from patients with noninvasive BC (Fig. 2B) and weak staining was found in paracancer samples (Fig. 2A). The positive expression of iASPP was more frequent in 89 of 98 (90.8%) cases of invasive bladder cancer than in noninvasive bladder cancer (20 of 46; 43.5%) or paracancer samples (0 of 31; 0.0%). The difference was statistically significant (Fig. 2D).

3.5. The relationships between iASPP expression and clinicopathological factors

The correlation between iASPP expression level and a series of clinicopathological characteristics, including age, gender, tumor stage, histological grade, and lymph node metastasis, was conducted. As shown in Table 1, High expression of iASPP was significantly correlated with tumor stage ($P < .001$), histological grade ($P < .001$), and lymph node metastasis ($P < .01$), but not correlated with the patient's age ($P = .607$) and sex ($P = .820$).

3.6. The relationships between iASPP expression and 5-year OS rate

The median 5-year OS of iASPP-positive patients was 49 months (95% confidence interval [CI]: 45.8–52.6 months). The median 5-year OS of iASPP-negative patients was 58 months (95% CI: 55.5–59.8 months). The 5-year OS rate was significantly shorter for iASPP-positive patients than for iASPP-negative patients; the difference was statistically significant (Fig. 3). A high level of iASPP expression was associated with decreased survival time.

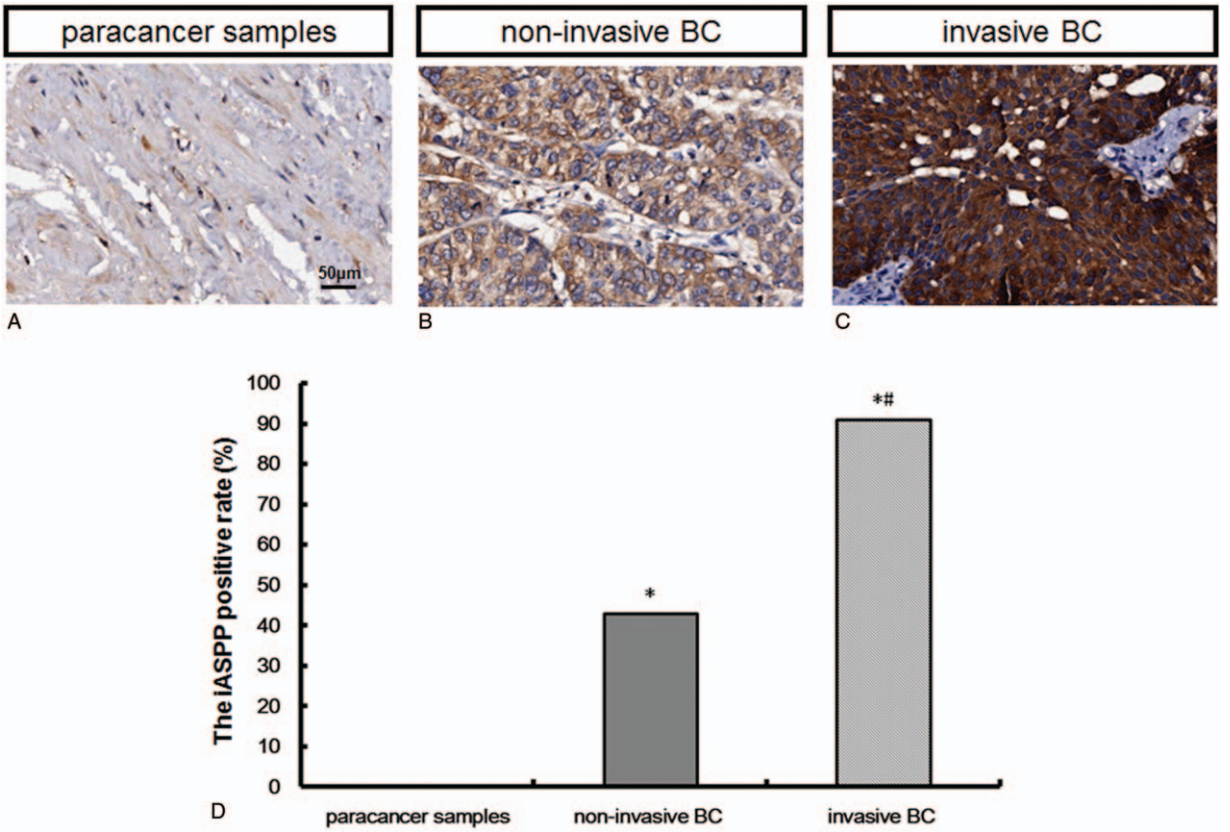

Figure 2. TMA-IHC assessment of iASPP expression. Strong cytosolic staining was found in cancer samples from patients with invasive BC (C), moderate cytosolic staining was found in cancer samples from patients with non-invasive BC (B) and weak staining was found in paracancer samples (A). iASPP positive rate of invasive BC was highest, and iASPP positive rate of noninvasive BC was higher than that of paracancer samples (D). *vs. paracancer samples, $P < .05$; #vs. noninvasive BC, $P < .05$. BC = bladder cancer, iASPP = inhibitory member of the apoptosis-stimulating protein of p53, RT-PCR = real-time polymerase chain reaction, TMA-IHC = tissue microarry immunohistochemistry.

4. Discussion

Human iASPP, which is encoded by PPP1R13L located on 19q13.2–3,[23] has 2 isoforms (407 and 828 amino acids-aa).[7,24] The shorter form of iASPP (407 aa) is a nuclear protein, and the longer form of iASPP (828 aa) is located in both the nucleus and cytoplasm.[7,23,24] iASPP was considered as an oncogene that not only inhibits the transcriptional activity of p53 on promoters of downstream genes, but also promotes carcinogenesis through p53-independent mechanisms, mainly by inhibiting the apoptotic activity of p63 and p73.[25] Several lines of direct evidence demonstrated a significant association between iASPP expression and poor survival, tumor grade and lymph node metastasis in head and neck cancer,[11] oral squamous cell carcinoma,[12] cervical cancer,[13] ovarian cancer,[14] and endometrial cancer.[15]

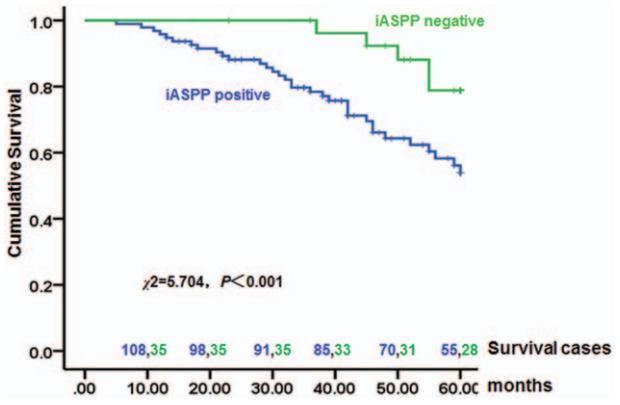

Figure 3. Kaplan-Meier curve for comparison of 5-year OS between patients with iASPP-positive patients with iASPP-negative patients. The 5-year OS rate was significantly shorter for iASPP-positive patients than for iASPP-negative patients. A high level of iASPP expression was associated with decreased survival time. BC = bladder cancer, iASPP = inhibitory member of the apoptosis-stimulating protein of p53, OS = overall survival.

However, little is known about the role of iASPP in BC. In this study, we explored the expression patterns of iASPP and relationship with clinical pathologic characteristics in BC. Our data showed iASPP mRNA and protein was overexpressed in cancer tissue of BC. iASPP signal intensity and positive percentage of invasive BC were highest, and that of noninvasive BC were higher than that of paracancer samples. Significant association observed between iASPP expression and lymph node metastasis indicated iASPP may be involved in the aggressive tumor growth or metastasis of BC. Metastasis consists of a series of sequential steps, all of which have to be accomplished. These include detachment of cells from a primary tumor, survival of cancer cells in the circulation, and arrest in the secondary sites.[26] It is well known that apoptosis is a rate-limiting process in the tumor metastasis cascade. iASPP could protect cancer cells against apoptosis by inhibiting the apoptotic activity of p53, p63, and p73,[25] which might confer metastatic properties to cancer cells. Our data demonstrate that iASPP is overexpressed in BC

and promotes the malignancy of BC. Whether will down-regulated expression iASPP reduce the degree of malignancy? Further studies are needed in the future.

Kaplan-Meier analysis showed that 5-year OS rate of iASPP-positive patients was significantly shorter than that of iASPP-negative patients. The result indicated survival time of patients with high level of iASPP expression decreased. It was reported the expressions of BRCA1, MDR1, and ERCC1 maybe as prognostic markers for poor survival in patients with BC.[27–29] However, the examinations of these markers had been performed using RT-PCR, which require complicated procedures, none of them is presently in clinical use. In the present study, the expression of iASPP was detected by immunohistochemical analysis that can be applied in almost every pathology laboratory. This finding may be widely applicable in clinical practice.

However, further studies with a large number of patients are warranted to confirm this result. In this study, liquid chromatography tandem mass spectrometry, RT-PCR and Western blot were only performed in invasive BC tissue for enough samples. In the following study, it should be performed in invasive and noninvasive BC at the same time. In this study, patients were roughly divided into 2 groups based on 70 years' old. Whether iAPPS expression is age-related is worth further study in detail.

In conclusion, our research indicates that iASPP is overexpressed in BC and promotes the malignancy of BC. iASPP may be a prognostic marker for BC and may serve as a potential therapeutic target for BC.

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