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Elevated expression of iASPP in head and neck squamous cell carcinoma and its clinical significance

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Abstract iASPP is shown to be elevated in several cancers. However, the role of iASPP in head and neck squamous cell carcinoma (HNSCC) remains unknown. We have investigated iASPP expression in HNSCC tissue and cell lines and evaluated its prognostic significance in HNSCC. The expression of iASPP in 109 primary HNSCC tissue specimens was examined by immunohistochemistry and its association with clinicopathological parameters and prognosis was analyzed. Additionally, expression status of iASPP in 16 paired HNSCC tissues and 7 HNSCC cell lines was evaluated by quantitative real-time PCR (qPCR) and immunoblotting. The protein and mRNA expression of iASPP were increased in HNSCC tissues and cell lines. Immunohistochemical staining indicated iASPP was detected in both cytoplasm and nucleus. Importantly, overexpression of cytoplasmic and nuclear iASPP was significantly associated with T classification ($p = 0.002$ and $p = 0.033$, respectively), clinical stage ($p < 0.001$ and $p = 0.004$), lymph node metastasis ($p = 0.001$ and $p < 0.001$), and recurrence (both $p < 0.001$). Survival analysis demonstrated high iASPP expression significantly correlated with shorter disease-free survival (DFS) (both $p < 0.001$ for cytoplasmic and nuclear expression) and overall survival (OS) (both $p < 0.001$ for cytoplasmic and nuclear expression). Multivariate analysis revealed that cytoplasmic iASPP was the only independent prognostic factor for HNSCC patients. iASPP expression is elevated in HNSCC tissues and cell lines, which suggests iASPP may contribute to the malignant progression of HNSCC, and serves as a novel prognostic marker and a potential therapeutic target in HNSCC.

Keywords IASPP · HNSCC · Metastasis · Apoptosis · Prognosis

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Introduction

Head and neck squamous cell carcinoma (HNSCC) represents a seriously clinical problem with more than 500,000 new cases diagnosed [1] and 200,000 deaths worldwide annually [2]. Despite advances in diagnosis and treatment, HNSCC is still a great threat to human life with a 5-year survival of only 50 % [3]. Like all solid tumors, HNSCC is thought to be initiated and progress through a series of genetic alterations. Therefore, identifying common genetic alterations which occur during HNSCC progression and novel biomarkers is an important component for early detection and further successful prevention and treatment for patients with HNSCC [4, 5].

The apoptosis stimulating protein of p53 (ASPP) family, including 3 members, ASPP1, ASPP2, and iASPP, was identified as specific regulators of p53-, p63-, and p73-mediated apoptosis [6]. Among them, ASPP1 and ASPP2 are specific activators of all three p53 family members p53,

p63, and p73, increasing the transactivation of proapoptotic genes [7, 8]. In contrast, iASPP, an evolutionally conserved inhibitory member of the ASPP family, can specifically inhibit p53-mediated cell apoptosis, and its overexpression confers resistance to ultraviolet radiation and cisplatin-induced apoptosis in cultured cells. Also, it is considered as an oncoprotein that cooperates with Ras, E1A, and E7, but not mutant p53, to transform cells in vitro [9]. Furthermore, iASPP overexpressed in several kinds of human cancers, such as breast carcinomas [9], acute leukemia [10], hepatocellular carcinoma [11], and ovarian cancer [12]. Elevated iASPP expression contributes to tumorigenesis by proliferative and antiapoptotic effects [13–17]. Thus, iASPP might be a potential molecular target for cancer therapy. However, there is little information available in the literature about the role of iASPP expression in HNSCC, particularly with respect to the prognosis. Therefore, in order to elucidate the clinical significance of iASPP in HNSCC, the present study was undertaken to investigate iASPP expression pattern in HNSCC tissue samples and cell lines, and further to assess the relevance between iASPP expression and clinicopathological parameters and prognosis in HNSCC.

Materials and methods

Patients and specimens

In our study, tissue specimens were obtained from 109 patients with HNSCC who underwent partial or total laryngectomy at the Department of Otolaryngology of Xiangya Hospital in Central South University from January 2002 to May 2005. None of them had previous malignancies history or treatment such as radiotherapy or chemotherapy. The main clinicopathological information of all patients was described in Table 1. There were 106 male and 3 female patients, with a median age of 57 years (range 27–79 years, standard deviation (SD) = 9.898). The carcinomas were distributed as follows: 99 larynx carcinomas (including 28 cases of the supraglottis, 70 cases of the glottis, and 1 case of the subglottis) and 10 hypopharyngeal carcinomas. According to the 2002 TNM classification of malignant tumors by the International Union Against Cancer [18], 16 patients were in stage I, 26 in stage II, 34 in stage III, and 33 in stage IV. Considering pathological grading, 67 cases were classified as well differentiated (G1), 32 as moderately differentiated (G2), and 10 as poorly differentiated (G3). Forty-three patients had lymph node metastasis and sixty-three patients experienced tumor recurrence after surgery.

In addition, 16 paired snap-frozen samples of HNSCC and their adjacent non-tumor tissues from the patients with HNSCC were collected for total RNA and protein

Table 1 Clinicopathologic characteristics of 109 HNSCC patients

Variables	Number of patients	Percentage
Age		
<57	55	50.5
≥57	54	49.5
Sex		
Female	3	2.8
Male	106	97.2
Alcohol intake		
Yes	59	54.1
No	50	45.9
Smoking		
Yes	70	64.2
No	39	35.8
Tumor site		
Superaglottic	28	25.7
Glottic	70	64.2
Subglottic	1	0.9
Hypopharyngeal	10	9.2
Tumor grade		
G1	67	61.5
G2	32	29.4
G3	10	9.2
Tumor size		
T1	20	18.3
T2	38	34.9
T3	34	31.2
T4	17	15.6
Clinical stage		
I	16	14.7
II	26	23.9
III	34	31.2
IV	33	30.3
Lymph node metastasis		
N0	66	60.6
N+	43	39.4
Recurrence		
Yes	63	60.6
No	41	39.4

extractions. Informed consent was obtained from all patients before surgery, and the study was approved by the Research Ethics Committee of Central South University, Changsha, China.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissues were immunostained using the PV-6001 Two-Step IHC Detection Reagent following the manufacturer's instruction (Zhong-Shan Goldenbridge Bio, Beijing, China), as previously

described [19, 20]. The iASPP antibody was a rabbit polyclonal antibody (ab-34898, dilution 1:1,600; Abcam, Cambridge, USA). A sample probed with normal goat serum instead of primary antibody under the same experimental conditions was used as negative control.

Immunohistochemical evaluation was performed by two independent and experienced pathologists (Li Xiang and Feng Xueping), who were blinded to the clinicopathological parameters of the sections. A score index (values 0–9), obtained as a product of staining intensity (0–3: absent, 0; weak, 1; moderate, 2 and; strong, 3) and percentage of immunopositive tumor cell (absent, 0; < 10 %, 1; 10–50 %, 2 and; > 50 %, 3). Staining in cytoplasm and nucleus was evaluated separately. For statistical analyses, the expression of iASPP was thus divided into low expression group (scored ≤ 4) and high expression group (scored ≥ 6) [21].

Cell culture

The HNSCC cell line Tu686 was established from a primary tumor in the base of tongue. Derived through repeated in vivo selection in nude mice from a lymph node metastasis from the same patient, M2 and M4 were highly metastatic cell lines capable of generating high incidences of lymph node and lung metastasis [22]. The above 3 HNSCC cell lines were kindly provided by Dr. Zhuo (Georgia) Chen (Emory University Winship Cancer Institute, Atlanta, Georgia). Additional HNSCC cell lines HNE1, HNE2, CNE1, and CNE2 were derived from nasopharyngeal carcinoma and kindly provided by Cancer Research Institute, Xiangya School of Medicine, Central South University, Changsha. Tu686, M2, and M4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1:1). HNE1, HNE2, CNE1, and CNE2 cells were cultured in RPMI 1640 medium. Both media were supplemented with 10 % fetal bovine serum (FBS) and antibiotics in a humidified atmosphere with 5 % CO_2 at 37 °C.

Quantitative real-time RT-PCR analysis

Total RNA was extracted from frozen tissues using Trizol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized with the Revert Aid First Strand cDNA Synthesis Kit (FERMENTAS, USA). Real-time PCR was performed on an MiniOpticon Real-Time PCR System using SYBR green Master Mixture. The primers for iASPP and GADPH were as follows: iASPP (180 bp), 5′-GAAAGCCTGGAACGAGTCTG-3′ (forward) and 5′-GCGCTAGTGAGGTTGTCCTT-3′ (reverse); GADPH (179 bp), a endogenous control, 5′-C GACCACTTTGTCAAGCTCA-3′ (forward) and 5′-ACTG AGTGTGGCAGGGACTC-3′ (reverse). The mRNA

expression levels were normalized against GADPH and determined by $2^{-\Delta\Delta CT}$ method [23]. Each sample was run in triplicate.

Western blotting

The protein of oral epithelial origin, premalignant-Dysplastic Oral Keratinocyte (DOK) cell line was kindly provided by Xiangya School of Stomatology, Central South University, Changsha. Total protein lysates, harvested from tissue specimens and 7 HNSCC cell lines, together with DOK protein, were quantified by BCA protein assay. Western blotting was performed as previously described [20]. The same amount of proteins (50 μg) was separated by 10 % SDS–PAGE, and the dilution of iASPP antibody (ab-34898) was 1:1,500. The experiments were repeated three times.

Follow-up

Apart from 5 patients lost to follow-up, 104 patients had complete follow-up data after surgery. Recurrence and metastasis were determined by clinical examination, imaging evaluation, and pathological studies. OS and DFS time were measured from the date of surgery to the date of death or tumor recurrence. Deaths from other causes were treated as censored cases. The follow-up time ranged from 2 to 60 months, with a mean follow-up time of 42.74 months (SD = 20.817).

Statistical analysis

Continuous variables were expressed as mean ± SD. Student's two-tailed t test was applied to compare data between groups. Statistical significance between iASPP expression and clinicopathological parameters was analyzed by the χ^2 test. Survival curves were calculated according to Kaplan–Meier method and compared with the log-rank test. Multivariate analysis was done using the Cox's proportional hazards model to identify independent prognostic factors. All statistical analyses were performed with SPSS 19.0 software. $p < 0.05$ was considered statistically significant.

Results

Overexpression of iASPP protein in HNSCC tissue and cell levels

iASPP immunoreactivity was observed predominantly throughout the cytoplasm of HNSCC tumor cells and also detected in the nucleus. Its staining was diffuse (Fig. 1). High cytoplasmic and nuclear expression of iASPP were detected in 62 (56.9 %) and 30 (27.5 %) primary tumor
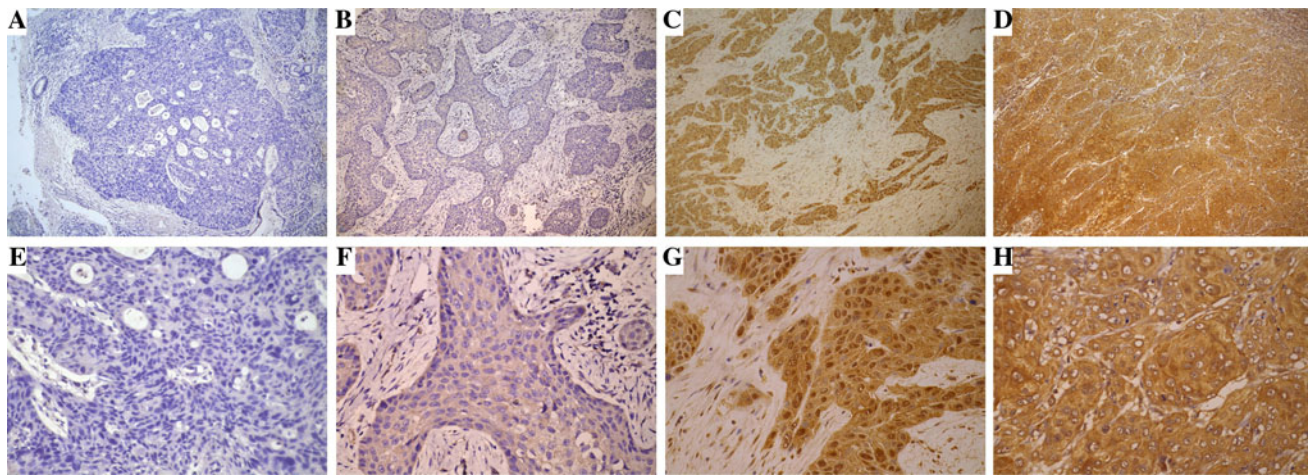
Fig. 1 Representative images from immunohistochemical staining of iASPP in primary HNSCC tissues. Negative control of iASPP in HNSCC tissues (**a, e**); low cytoplasmic and nuclear iASPP expression (**b, f**); high coexpression of cytoplasmic and nuclear iASPP (**c, g**); strong cytoplasmic and weak nuclear iASPP expression (**d, h**). (original magnification **a–d**, ×100; **e–h**, ×400)

Fig. 2 Western blotting analyses of iASPP protein expression in the 7 selected human HNSCC cell lines and premalignant-dysplastic oral keratinocyte (DOK) cell line (used as a control) (**a**); the abundance of iASPP protein was shown relative to the levels of β-actin protein, which demonstrated iASPP was up-regulated in HNSCC cell lines when compared with DOK (**b**)
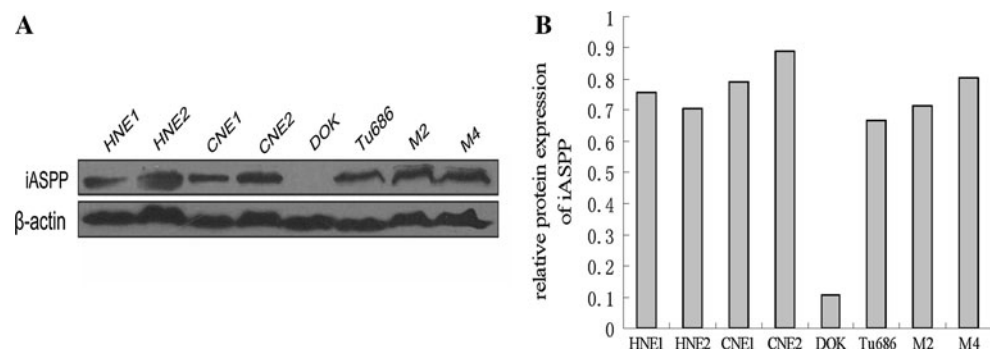
samples, respectively, while high levels of coexpression of cytoplasmic and nuclear iASPP staining were observed in 30 (27.5 %) primaries. Moreover, by Western blotting analysis, increased expression of iASPP protein was detected in all the selected 7 HNSCC cell lines (HNE1, HNE2, CNE1,CNE2, Tu686, M2, M4) when compared with DOK cell line, a control which demonstrated almost negative iASPP protein expression (Fig. 2a). Relative iASPP expression levels in HNE1, HNE2, CNE1,CNE2, Tu686, M2, M4, and DOK were 0.76, 0.70, 0.79, 0.89, 0.67,0.71, 0.80, and 0.11 units (iASPP/β-actin protein), respectively (Fig. 2b).

**iASPP mRNA and protein expression in paired HNSCC tissues**

In order to get to know better the expression pattern of iASPP mRNA and protein, 16 matched clinical frozen HNSCC specimens were collected for further investigation. qPCR and immunoblotting analysis demonstrated that the

mRNA and protein levels of iASPP were significantly elevated in cancers compared to the adjacent non-tumor tissues ($p < 0.001$ and $p = 0.002$, respectively; Fig. 3a, b).

Association of increased iASPP protein expression with clinicopathologic characteristics

The χ^2 test indicated cytoplasmic iASPP expression was significantly associated with HNSCC T classification ($p = 0.002$), clinical stage ($p < 0.001$), lymph node metastasis ($p = 0.001$), and recurrence ($p < 0.001$), respectively. And the significant difference was also found between overexpression of nuclear iASPP and tumor T classification ($p = 0.033$), advanced stage ($p = 0.004$), lymph node metastasis ($p < 0.001$), and recurrence ($p < 0.001$). However, other variables such as age, alcohol history, smoking, tumor grade, and tumor site showed no statistically significant associations with iASPP expression status (all $p > 0.05$; summarized in Table 2).

Fig. 3 Levels of iASPP mRNA and protein in 16 paired human HNSCC and adjacent non-tumor tissues. **a** Representative Western blotting results; **b** Relative expression levels of iASPP mRNA and protein in paired tissue samples, determined by *q*PCR and Western blotting analysis, respectively. The Student's *t* test demonstrated that iASPP mRNA and protein expressions in HNSCC tissues were significantly higher than those in PNTS (*$p < 0.001$ and **$p = 0.002$, respectively). The abundance of iASPP mRNA and protein was shown relative to the levels of GAPDH mRNA or β-actin protein, respectively. *T* tumor tissue, *N* non-tumor epithelium, *PNTS* paired non-tumor tissues
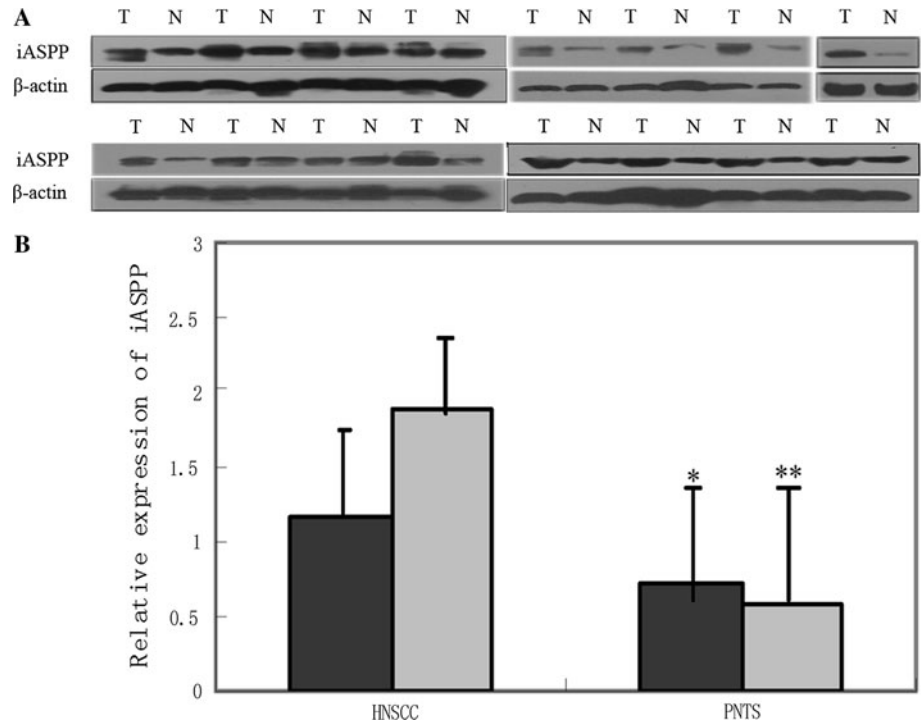
Survival analysis

To assess whether elevated iASPP expression correlates with worse prognosis, Kaplan–Meier survival curves were constructed using DFS and OS. The data revealed that a high level of cytoplasmic and nuclear iASPP expression in HNSCC was significantly associated with earlier disease recurrence (both $p < 0.001$; Fig. 4a, b) and shorter overall survival (both $p < 0.001$; Fig. 4c, d). Furthermore, a multivariate Cox regression analysis including age, grade, tumor site, T classification, clinical stage, metastasis, smoking, alcohol intake, cytoplasmic iASPP expression, and nuclear iASPP expression was performed to find out the independent prognostic factors. The results showed that cytoplasmic iASPP expression was the only independent prognostic factor associated with DFS (hazard ratio = 2.820, 95 % CI = 1.429–5.565, $p = 0.003$) and OS (hazard ratio = 4.273, 95 % CI = 1.903–9.594, $p < 0.001$) in patients with HNSCC after tumor resection (Table 3).

Discussion

To the best of our knowledge, the present study is the first report of elevated iASPP expression at both protein and mRNA levels in HNSCC tissue and cell lines. The results of qPCR and immunoblotting revealed that iASPP overexpressed in HNSCC tissues at both mRNA and protein levels when compared with adjacent non-tumor tissues.

Both cytoplasmic and nuclear iASPP expression status significantly correlated with T classification, clinical stage, recurrence, and lymph node metastasis. Moreover, both cytoplasmic and nuclear iASPP overexpression were significantly associated with earlier disease recurrence and worse survival outcome. Nevertheless, only cytoplasmic iASPP expression was the independent negative prognostic factor in HNSCC. All these findings imply that iASPP might play an important role in mediating the malignant progression of HNSCC and serve as a prognostic marker that predicts the clinical outcome in HNSCC.

Accumulated evidence suggests that iASPP highly expresses in several cancers such as breast cancer [9], acute leukemia [10], hepatocellular carcinoma [11], and ovarian cancer [12]. Our results that iASPP overexpressed in tumor tissues and cells were in line with the previous studies. We found the positive staining of iASPP in the majority of the HNSCC samples. Its expression appears to be predominantly in the cytoplasm, while nuclear immunoreactivity was also detected and iASPP overexpression was found in both nucleus and cytoplasm. In other malignancies, including hepatocellular carcinoma [11], non-small cell lung cancer [16], and prostate cancer [13], iASPP expression was only observed in the cytoplasm. Although in ovarian cancer [12], nuclear immunoreactivity was also detected in some cancer samples. The difference in the intracellular localization between our findings and others may due to the specificity of the anti-iASPP antibody we used is against both isoform 1 and isoform 2 of iASPP, which is predominantly a cytoplasmic protein (isoform 1)

Table 2 Correlations between iASPP expression and clinicopathologic variables in HNSCC patients

Variables	N	Cytoplasmic iASPP expression			Nuclear iASPP expression		
		Low	High	p*	Low	High	p*
Age							
<57	55	23	32	0.782	37	18	0.220
≥57	54	24	30		42	12	
Alcohol intake							
Yes	59	24	35	0.576	40	19	0.235
No	50	23	27		39	11	
Smoking							
Yes	70	28	42	0.378	47	23	0.095
No	39	19	20		32	7	
Tumor grade							
G1	67	28	39	0.724	48	19	0.805
G2 + G3	42	19	23		31	11	
Tumor site							
Glottic	70	33	37	0.256	54	16	0.144
Others	39	14	25		25	14	
T classification							
T1 + T2	58	33	25	0.002	47	11	0.033
T3 + T4	51	14	37		32	19	
Clinical stage							
I–II	42	27	15	<0.001	37	5	0.004
III–IV	67	20	47		42	25	
Lymph node status							
N0	66	37	29	0.001	56	10	<0.001
N+	43	10	33		23	20	
Recurrence[a]							
Yes	63	12	51	<0.001	36	27	<0.001
No	41	31	10		38	3	

* p < 0.05 was considered to be statistically significant

[a] Five patients lost to follow-up because of telephone number changes or home moving

but can also be found in the nucleus (isoform 2). And our findings were just consistent with the evidence which demonstrated when iASPP overexpressed, and the amount of nuclear iASPP was increased [24]. Furthermore, Our results were in accordance with previous studies of other malignancies, such as hepatocellular carcinoma, ovarian cancer, and endometrial endometrioid adenocarcinoma [25], in which the expression of iASPP was elevated in tumor tissues and a correlation between iASPP expression status was significantly correlated with tumor malignant phenotype, such as metastasis [25] and recurrence [11, 12]. In this respect, iASPP can be proved to be an oncoprotein and its overexpression contributes to tumor progression resulting from promoting migration, proliferation, antiapoptosis, invasion, and metastasis. There was no controversy that iASPP could promote cell proliferation and inhibit apoptosis. With regard to metastasis, the findings of recent studies are conflicting. iASPP was reported to be associated with lymph node metastasis in endometrial endometrioid adenocarcinoma [25], and another study indicated iASPP could promote cell migration and may be important for tumor metastasis [26]. However, expression of iASPP in ovarian cancer had no significant difference between the primary carcinomas and their metastatic foci. This may be due to the divergent iASPP evaluation methods between the two studies (primary carcinomas to primary carcinomas vs primary carcinomas to metastasis foci). Further investigations into the possible metastasis mechanisms are required.

Many mechanism researches have explained the role of iASPP in tumorigenesis and progression. Firstly, it is noteworthy that iASPP binds to the protein p53 inhibiting its proapoptotic function and promotes cell growth and proliferation [27, 28], whereas recent studies indicate the role of iASPP in influencing apoptosis and modulating proliferation is irrespective of p53 status [13, 14]. Recently, it was reported that a p53-derived peptide that targets iASPP can effectively trigger tumor cell death by a p73-dependent mechanism. These findings promoted further study of iASPP targeting as a therapeutic strategy to activate p73 [27]. Secondly, iASPP was found to be up-regulated by the activation of NF-κB factors in tumors, rather than the epigenetic alteration, which was involved in the down-regulation of ASPP1 and ASPP2 genes [7, 29, 30]. Therefore, iASPP may serve as a mediator between the activation of NF-κB and the inhibition of the proapoptotic function of p53. Thirdly, iASPP acts as an oncogene that can extensively enhance the transforming activity of RAS and E1A oncogenes in culture of rat and mouse embryo fibroblasts [9, 26]. Finally, a recent study identifies iASPP as a new binding partner of PP1, interacting through a noncanonical PP1 binding motif [31]. One can speculate that iASPP-binding targets them for proteasome degradation, while the specific mechanism between iASPP and PP1 needs further exploration.

Finally, based on the Kaplan–Meier survival analysis, Overexpression of cytoplasmic and nuclear iASPP had significant adverse effect on DFS and OS, which had been verified in both ovarian cancer and hepatocellular carcinoma. Moreover, in multivariate analysis, only cytoplasmic iASPP expression was found to be a negative independent prognostic indicator in HNSCC, which has been established in ovarian cancer.

In conclusion, our current study indicates that iASPP is elevated in human HNSCC and iASPP overexpression significantly correlates with tumor malignant progression and poor survival in HNSCC. These results suggest that iASPP may serve as a specific and novel prognostic marker
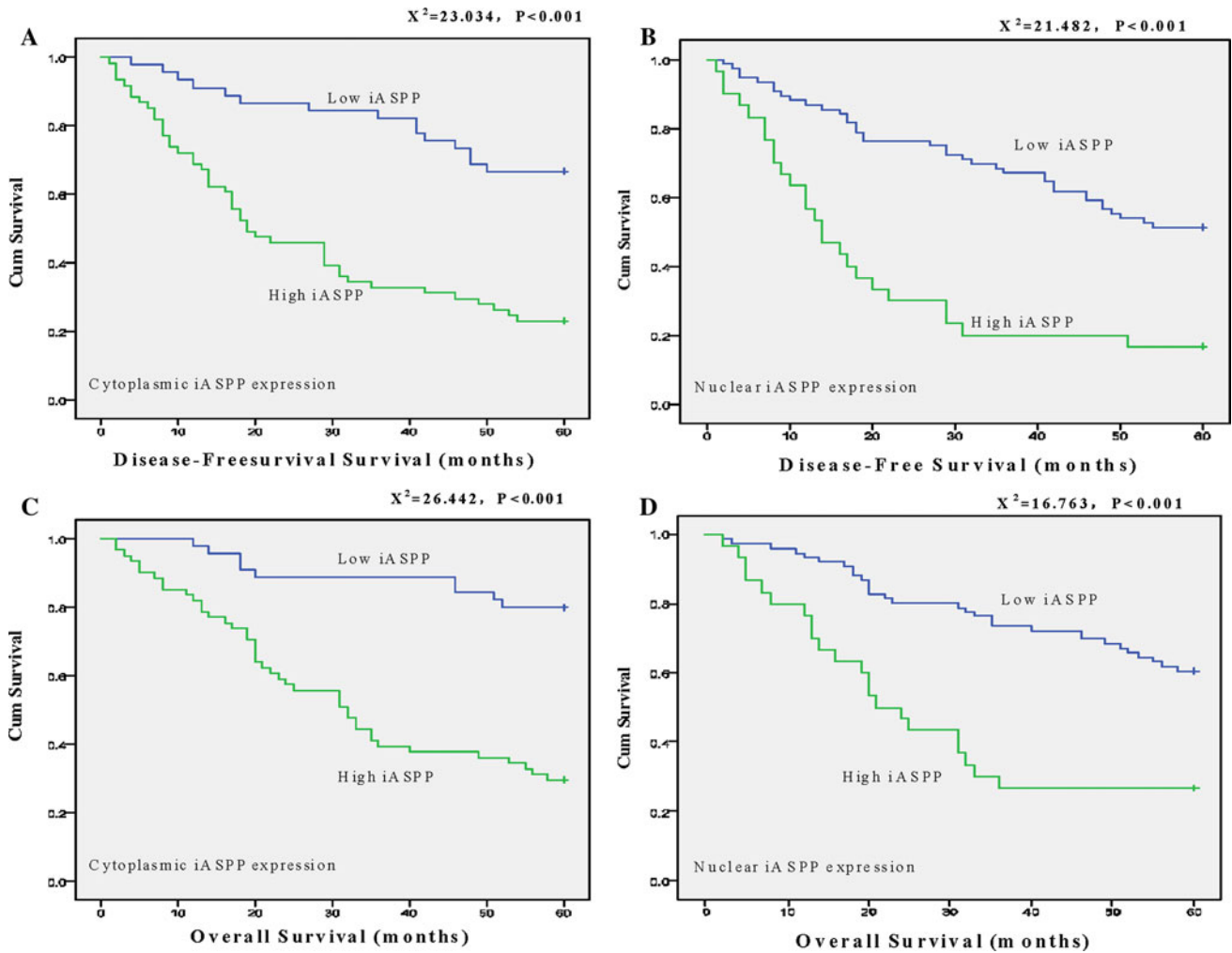
Fig. 4 Kaplan–Meier survival analysis for iASPP. Disease-free survival for cytoplasmic (**a**) and nuclear iASPP expression (**b**); overall survival for cytoplasmic (**c**) and nuclear iASPP expression (**d**). The log-rank test was used to calculate *P* value

Table 3 Multivariate Cox model analysis of disease-free and overall survival

Factor	Disease-free survival		Overall survival	
	Hazard ratio (95 % CI)	*p*	Hazard ratio (95 % CI)	*p*
Age (<57/≥57)	1.062 (0.622–1.815)	0.825	1.041 (0.576–1.880)	0.894
Alcohol intake (yes/no)	0.684 (0.406–1.154)	0.155	0.748 (0.419–1.336)	0.326
Smoking (yes/no)	1.578 (0.864–2.881)	0.137	1.426 (0.741–2.743)	0.288
Tumor grade (G1/G2 + G3)	1.438 (0.829–2.494)	0.196	1.488 (0.807–2.742)	0.203
Tumor site (glottic/others)	0.818 (0.428–1.563)	0.543	0.692 (0.340–1.409)	0.310
T classification (T1 + T2/T3 + T4)	0.810 (0.369–1.775)	0.598	1.162 (0.486–2.779)	0.737
Clinical stage (I–II/III–IV)	1.420 (0.549–3.676)	0.470	0.995 (0.325–3.045)	0.993
Lymph node status (N0/N +)	1.381 (0.652–2.924)	0.399	1.386 (0.592–3.247)	0.452
Cytoplasmic iASPP expression (high/low)	2.820 (1.429–5.565)	**0.003**	4.273 (1.903–9.594)	**0.000**
Nuclear iASPP expression (high/low)	1.595 (0.865–2.943)	0.135	1.213 (0.634–2.321)	0.560

All the clinicopathological variables listed in table were enrolled in the multivariate analysis

p values in bold were statistically significant

95 % CI 95 % confidence interval

in HNSCC. Furthermore, considering the findings that inhibiting iASPP activity presents negative effect on tumorigenesis and improves chemosensitivity in vitro reported by others [11, 12], iASPP might also serve as an attractive therapeutic target in HNSCC. However, further investigation into elucidation the molecular mechanism of iASPP involved in HNSCC progression and prognosis will be necessary, which may promote new approaches of iASPP targeting as a therapeutic strategy for effective tumor management.

Conflict of interest We declare that we have no conflict of interest.

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