Correlation of Expression of Phosphorylated and Non-phosphorylated Yes-associated Protein with Clinicopathological Parameters in Esophageal Squamous Cell Carcinoma in a Korean Population

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Abstract. *Yes-associated protein (YAP) is a nuclear downstream effector of the Hippo pathway, a regulator of cell growth. The phosphorylated form of YAP (pYAP), located in the cytoplasm, prevents cellular proliferation by spatially segregating YAP from the nucleus. This study aimed at investigating the relationships of pYAP and YAP with clinicopathological variables in esophageal squamous cell carcinoma (ESCC). Samples of ESCC from 142 patients were studied using immunohistochemistry for YAP, pYAP and Ki-67. In all cases of ESCC, higher nuclear expression of YAP was correlated with Ki-67 expression, tumor diameter, histological grade (1-2 versus 3), and pathological TNM stage (I versus II–IV) in univariate analyses (p=0.036, p=0.025, p=0.021, and p=0.033, respectively). Higher nuclear expression of YAP was associated with worse overall and disease-free survival (p=0.006 and p=0.008, respectively). Multivariate analysis showed higher nuclear expression of YAP to be an independent prognostic marker for overall survival (p=0.034). We observed a trend towards inverse correlation of cytoplasmic pYAP expression and histological grade (1-2 versus 3) (p=0.087). Our results suggest that YAP shifts from the nucleus to the cytoplasm as a consequence of phosphorylation, which occurs in the presence of high tumor cell density in the case of ESCC, and may be a potential indication of histological differentiation. Nuclear expression of YAP is correlated with tumor cell proliferation and is an independent predictor of worse prognosis of ESCC.*

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Yes-associated protein (YAP) is a negative regulator of the mammalian Hippo pathway and a candidate human oncogene located at chromosome 11q22 (1, 2). The Hippo pathway is known as a tumor suppressor pathway in mammals and *Drosophila*. At low cell densities, YAP localizes in the nucleui of NIH3T3 cells, where it interacts with a number of transcription factors (3). The function of YAP as a transcriptional co-activator is to promote cell proliferation and inhibit apoptosis (3-5).

The mammalian Hippo pathway consists of a kinase cascade in which mammalian sterile twenty-like 1/2 (MST1/2) (Ste-20 family of protein kinases; Hpo in *Drosophila*) phosphorylates the protein kinase nuclear DBF-2-related kinase (LATS1/2) (Wts in *Drosophila*) (6). In environments of high cell density or protein–protein interactions, LATS1/2 has been shown to phosphorylate YAP *in vitro* (3, 7, 8). Phosphorylation of YAP, particularly at serine 127, generates a 14-3-3 binding site that leads to YAP moving from the nucleus and localizing to the cytoplasm (4, 8, 9). YAP becomes inactivated by relocalization and its ability to act as a co-activator is diminished (4, 10, 11). Recent studies have generally shown an increased expression of YAP in carcinoma compared with normal tissue (12). In hepatocellular carcinoma (HCC), YAP was shown to be an independent prognostic marker for overall and disease-free survival (13). In non-small cell lung carcinoma, YAP positivity was associated with a trend towards lower overall survival (14). Muramatsu *et al.* demonstrated that nuclear expression of YAP is associated with shortened overall survival in esophageal squamous cell carcinoma (ESCC) (15).

In this study, we investigated the expression pattern of YAP and phosphorylated YAP (pYAP) in normal esophageal squamous cells and ESCC by immunohistochemistry. We also evaluated the relationships between YAP and pYAP expression and clinicopathological variables and Ki-67 expression.

Materials and Methods

Patients, tissue samples, and reagents. We investigated 171 samples from 142 ESCC patients; 142 samples were of ESCC tissues and 29 were of normal esophageal tissues (tumor-adjacent normal squamous epithelium). Information on the patients and samples was obtained from the surgical pathology files maintained at the Pathology Department of Chungnam National University Hospital and the Seoul Samsung Medical Center, South Korea between 1995 and 2004. All archival tissues were routinely fixed in 10% neutral-buffered formalin and embedded in paraffin. To create a tissue microarray, tissue columns (3.0 mm in diameter) were punched from the original paraffin blocks and inserted into new paraffin blocks. Arrays were constructed using two 3-mm diameter cores for the tissues.

Histological grading. ESCCs were separated into three subgroups according to the degree of histological differentiation as follows: grade 1, well-differentiated; grade 2, moderately differentiated; and grade 3, poorly differentiated. Grade 1 cases were characterized by similarity to the stratified squamous epithelium of normal esophagus. Grade 3 cases were difficult to distinguish from other esophageal cancer subtypes.

Immunohistochemical analysis. Tissue sections embedded in the microslides were de-paraffinized with xylene and hydrated in serial solutions of alcohol. The sections were heated in a pressure cooker containing 10 mmol/l sodium citrate (pH 6.0) for 3 min at full power, for antigen retrieval. Peroxide blocking was performed using 3% H_2O_2 in methanol at room temperature for 10 min, and non-specific protein-binding sites were blocked by incubation with serum-free protein for 20 min. The sections were incubated overnight at 4˚C with the primary antibodies: rabbit polyclonal antibody to YAP (diluted 1:100; Cell Signaling Technology, Danvers, MA, USA); rabbit polyclonal antibody to phospho-YAP (Ser127) (1:100; Cell Signaling Technology), and mouse monoclonal antibody to Ki-67 (1:100; Dako, Glostrup, Denmark). After washing, samples were incubated in Dako REAL EnVision/horseradish peroxidase rabbit/mouse detection reagent for an additional 20 min at room temperature, followed by additional washing. After rinsing, the chromogen was developed for 2 min. The slides were then counterstained with Meyer's hematoxylin, dehydrated and topped with coverslips.

Evaluation of immunostained samples. All immunostained slides were digitally scanned using a scanscope (Aperio ScanScope CS System, Vista, CA, USA). The immunohistochemical staining was scored using digitally scanned files and light microscopes. YAP and pYAP expression was observed in the nucleus and cytoplasm of tumor cells and in normal epithelial cells. Expression of Ki-67 was observed in the nuclei with the exception of the nuclei of basal cells. We used a modification of the scoring system of Allred *et al*. for evaluating both the intensity of immunohistochemical staining and the proportion of stained epithelial cells (nuclear YAP, cytosolic pYAP and nuclear Ki-67 stainings were independently analyzed) (16). The intensity was scored as: 1, weak; 2, intermediate; or 3, strong. The proportional scores were: 0, 0; 1, >0 to 1/100; 2, >1/100 to 1/10; 3, >1/10 to 1/3; 4, >1/3 to 2/3; 5, >2/3 to 1. To generate the total immunohistochemistry score, the intensity score and the proportional score were multiplied for each specimen. For categorical analyses of nuclear YAP and cytosolic pYAP stainings, the immunoreactivity in tumor cells was graded as low or high from the mean values (Cut-off values were 4 and 2 for expressions of nuclear YAP and cytoplasmic pYAP, respectively). Each sample was examined separately and scored by two pathologists (K. H. K. and M. K. Y.). Discrepancies in scores were discussed to obtain a consensus.

Statistical analysis. For the statistical analysis of staining intensity, the data for each tissue were categorized as 'high' or 'low' from the mean score for nuclear and cytoplasmic expression of YAP and pYAP. The clinicopathological variables were analyzed for statistical significance using the Mann-Whitney *U*-tests and Pearson's χ^2 test. For the evaluation of survival, Kaplan-Meier survival curves were constructed with the log rank test. Multivariate survival analysis was performed using the Cox's proportional hazard model. Statistical significance was assumed when $p<0.05$ (SPSS 19; SPSS Inc., Chicago, IL, USA).

Results

Clinicopathological features and the expression patterns of YAP and pYAP in normal esophagus and ESCC. We investigated 142 samples of ESCC and 29 samples of normal esophageal tissue (tumor-adjacent normal squamous epithelium) from 142 patients with ESCC. The average age of the patients was 69.07 years, and the male:female ratio was 138:4 (97.2%:2.8%).

In normal esophageal epithelia, YAP was usually expressed in the nuclei of the basal cells (Figure 1A). Some of the normal epithelia exhibited faint cytoplasmic expression in the epithelial granular layer. In ESCC tissue, YAP was generally expressed in both the nucleus and cytoplasm, but was more strongly expressed in the nucleus (Figure 1B). pYAP was weakly expressed from the basal to superficial normal squamous cells in both nuclei and cytoplasm (Figure 1C). In the cytoplasm of ESCC tissues, pYAP was expressed more strongly and diffusely than in normal esophageal epithelia (Figure 1D). Some peritumoral fibroblasts, endothelial cells and lymphocytes exhibited nuclear and cytoplasmic expression of YAP and pYAP. Nuclear YAP expression, cytoplasmic pYAP expression and Ki-67 expression were higher in ESCC tissues than in normal esophageal squamous epithelia ($p=0.001$, $p=0.028$, and $p<0.001$, respectively; Table I). The Ki-67 expression was positively correlated with nuclear YAP expression, but was not correlated with cytoplasmic pYAP expression ($p=0.036$ and $p=0.780$, respectively; Table II).

Correlation of nuclear YAP and cytoplasmic pYAP expression with clinicopathological variables. Univariate analysis revealed that higher nuclear YAP expression was significantly associated with tumor diameter, Ki-67 expression, histological

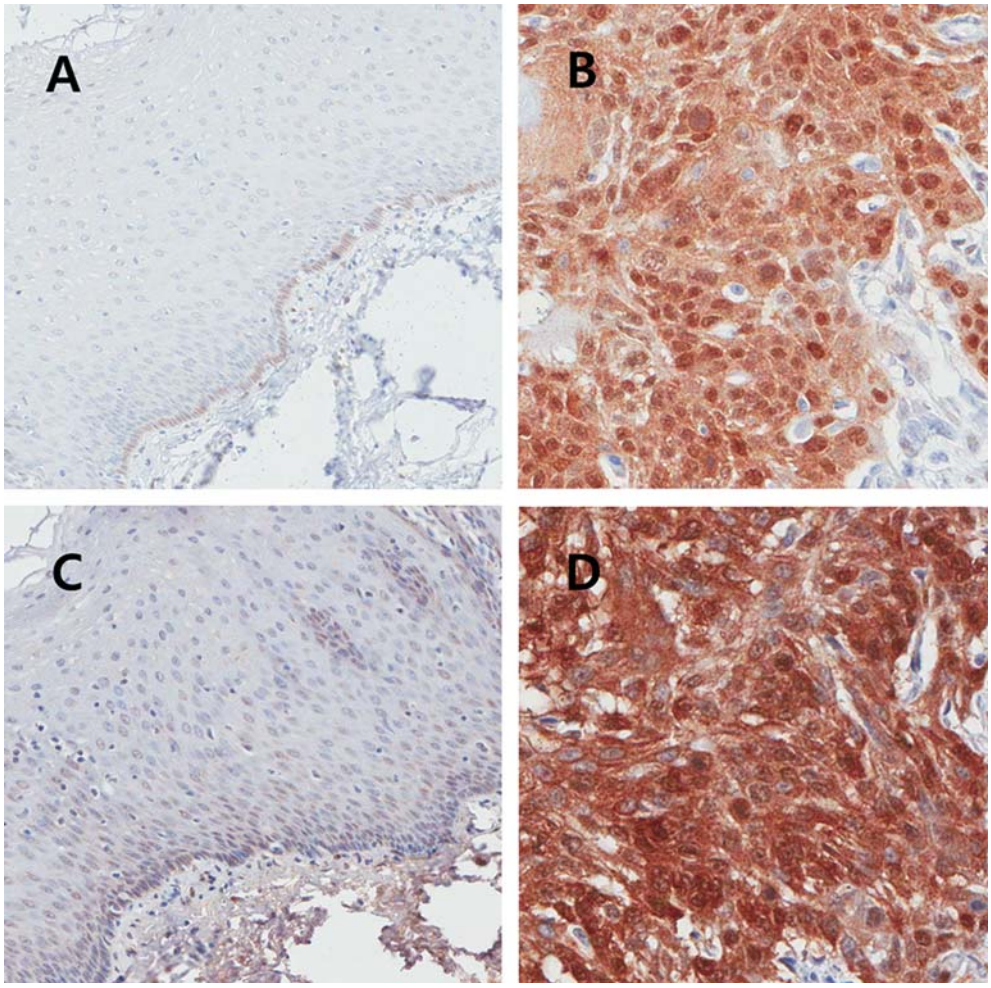

Figure 1. *Expression of Yes-associated protein (YAP) and phosphorylated YAP (pYAP) in esophageal squamous cell carcinoma (ESCC). A: Immunohistochemical staining of YAP in normal esophageal mucosa. YAP was usually expressed in the nuclei of the basal cells (YAP stain; original magnification, ×100). B: YAP exhibited strongly nuclear expression in ESCC (YAP stain; original magnification, ×400). C: Immunohistochemical staining of pYAP in normal esophageal mucosa. pYAP was weakly expressed from basal to superficial normal squamous cells in the nuclei and cytoplasm (pYAP stain; original magnification, ×100). D: pYAP exhibited strongly cytoplasmic expression in ESCC (pYAP stain; original magnification, ×400).*

grade (1-2 *versus* 3), pathological T category (I *versus* II-IV) and pathological TNM stage (stage I *versus* II–IV) (p=0.025, p=0.036, p=0.021, p=0.025, and p=0.033, respectively; Table II). Cytoplasmic expression of pYAP was associated with a trend towards lower histological grade (1-2 *versus* 3) (p=0.087; Table II).

We analyzed the relationships between nuclear expression of YAP and overall and disease-free survival rates. Kaplan–Meier survival estimates show that increased nuclear immunoreactivity for YAP in ESCC was significantly associated with shortened overall and disease-free survival (p=0.006 and p=0.008, respectively; Figure 2). Multivariate analysis of overall survival rates of ESCC showed nuclear YAP expression to be an independent prognostic factor (p=0.034; Table III).

Table I. *The expression profiles of nuclear Yes-associated protein (YAP), cytoplasmic phosphorylated YAP (pYAP) and nuclear Ki-67 in normal esophageal epithelium and esophageal squamous cell carcinoma.*

	Normal esophagus	Esophageal squamous cell carcinoma	*p*-Value
Nuclear YAP			0.001
No (%)	29 (7.6)	142 (92.4)	
Mean (SD)	1.45 (1.242)	3.60 (3.881)	
Cytoplasmic pYAP			0.028
No (%)	26 (10.1)	138 (89.9)	
Mean (SD)	3.77 (3.581)	6.31 (5.270)	
Ki-67 (range 0~15)			<0.001
No (%)	28 (1.5)	130 (98.5)	
Mean (SD)	0.64 (2.498)	9.39 (2.598)	

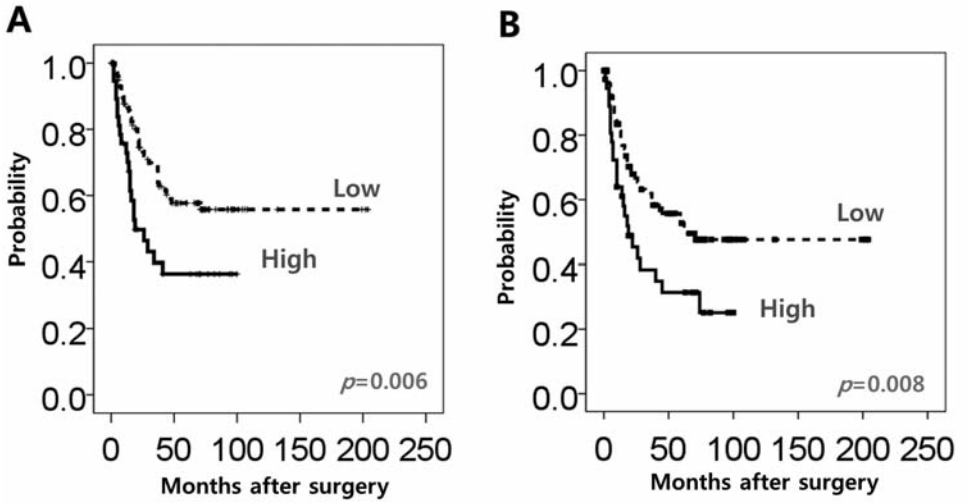

Figure 2. *Kaplan–Meier curves for overall survival rates of patients with esophageal squamous cell carcinoma according to the nuclear expression of Yes-associated protein (YAP) from immunohistochemistry. A: The nuclear YAP immunoreactivity of tumor cells was significantly associated with a worse overall survival (p=0.006, log rank test). B: Kaplan–Meier curves for disease-free survival according to the nuclear expression of YAP (p=0.008, log rank test).*

Table II. *Univariate analysis comparing nuclear expression of Yes-associated protein (YAP) and phosphorylated YAP (pYAP) with clinicopathological variables in esophageal squamous cell carcinoma.*

Characteristics	Nuclear YAP expression			Cytoplasmic pYAP expression		
	Low	High	*p*-Value	Low	High	*p*-Value
Age (year)			0.899[a]			0.184[a]
No.	104	38		84	54	
Mean (SD)	68.88 (8.88)	69.61 (9.61)		68.11 (8.11)	70.51 (8.42)	
Tumor diameter (cm)			0.025[a]			0.963[a]
No.	104	38		84	54	
Mean (SD)	3.968 (0.968)	5.097 (0.097)		4.332 (0.332)	4.243 (0.243)	
Ki-67 (range 0-15)			0.036[a]			0.780[a]
No.	93	37		77	49	
Mean (SD)	9.03 (0.030)	10.30 (0.30)		9.43 (.430)	9.43 (0.430)	
Histological grade, n (%)	104 (100)	38 (100)	0.021[b]	84 (100)	54 (100)	0.087[b]
1-2	91 (87.5)	27 (71.1)		65 (77.4)	48 (88.9)	
3	13 (12.5)	11 (28.9)		19 (22.6)	6 (11.1)	
Tumor stage, n (%)	104 (100)	38 (100)	0.025[b]	84 (100)	54 (100)	0.601[b]
I	26 (25.0)	3 (7.9)		17 (20.2)	9 (16.7)	
II-IV	78 (75.0)	35 (92.1)		37 (79.8)	45 (83.8)	
Pathological stage, n (%)	104 (100)	38 (100)	0.033[b]	84 (100)	54 (100)	0.852[b]
I	21 (20.2)	2 (5.3)		13 (15.5)	9 (16.7)	
II-IV	83 (79.8)	36 (94.7)		71 (84.5)	45 (83.3)	
Pathological stage, n (%)	104 (100)	38 (100)	0.390[b]	84 (100)	54 (100)	0.832[b]
I and II	55 (52.9)	17 (44.7)		42 (50)	28 (51.9)	
III and IV	49 (47.1)	21 (55.3)		42 (50)	26 (48.1)	

[a]Mann–Whitney *U*-test, [b]Pearson's χ^2 test.

Discussion

In this study, we investigated the expression patterns of YAP and pYAP in ESCC using immunohistochemistry. After immunohistochemical staining, we scored nuclear YAP and cytoplasmic pYAP staining separately and divided samples into low and high categories as some normal sqaumous epithelial cells also expressed nuclear YAP and cytoplasmic pYAP. As in previous studies by Muramatsu *et al.*, our study showed higher nuclear expression of YAP in ESCC to be

associated with a worse overall survival (15). Moreover, our results showed that higher nuclear expression of YAP correlated with a worse disease-free survival in Kaplan–Meier plots. Higher nuclear YAP expression correlated with tumor diameter, Ki-67 expression, histological grade (1-2 *versus* 3), pathological T category (I *versus* II-IV) and pathological TNM stage (stage I *versus* II-IV) in univariate analyses. Multivariate analysis, which included the potential confounding factors age, sex, experience of radiotherapy and chemotherapy, Ki-67 expression, cytoplasmic pYAP expression, T (tumor) stage, N (node) stage, and the level of histological differentiation (grade 1–3), showed high nuclear expression of YAP to be an independent prognostic factor (p=0.034; Table III).

In the Hippo pathway, initially identified by mosaic screens in *Drosophila*, loss of signaling leads to nuclear accumulation of YAP and increased activity of YAP as a transcription co-activator (17-20). In the Hippo pathway, MST1/2 phosphorylates LATS, which in turn phosphorylates YAP. pYAP can then follow one of two pathways. One is phosphorylation-dependent 14-3-3 binding and cytoplasmic retention to segregate it from the nucleus. The other involves subsequent ubiquitination and degradation by phosphorylation on serine 381 and its complete removal. Using a rabbit polyclonal antibody against pYAP (serine 127), and according to the two pathways described, we assume that pYAP immunostaining revealed most existing serine 127-pYAP because serine-381-pYAP can not accumulate because of its degradation (3, 4, 11, 21).

Many studies have been performed to evaluate YAP expression in human solid tumors and to identify the signal pathways that regulate YAP. YAP expression has been observed in many types of solid cancer, including HCC and lung cancer, colonic, ovarian and breast cancer. In particular, nuclear localization of YAP has been noted in the above types of cancer (12, 13). In patients with HCC, YAP expression is an independent prognostic marker for disease-free and overall survival (Kaplan-Meier and Cox regression). Xu *et al*. reported that immunohistochemically identified expression was predominantly observed in the nuclei of tumor cells, but cytoplasmic and nuclear expression was not scored separately (13). In 120 cases of ESCC, Muramatsu *et al*. separated the nuclear and cytoplasmic expression of YAP, and showed nuclear YAP expression, but not cytoplasmic expression, to be associated with worse overall survival (15). We previously reported that increased cytoplasmic expression of YAP is correlated with lower pathological TNM staging (stage I) in pulmonary squamous cell carcinoma. Increased nuclear expression of YAP in pulmonary adenocarcinoma was correlated with cyclin D1 expression (from multivariate analysis) (22).

In our current study, nuclear expression of YAP was higher in ESCC than in normal tissues. These results support the hypothesis that the excess production of YAP in the nucleus provides ESCC with growth signals (15). Cytoplasmic expression of pYAP in ESCC was higher than in normal squamous epithelia (p=0.028; Table I) and was correlated with a trend towards lower histological grade (1-2 *versus* 3) (p=0.087; Table II). Diffuse cytoplasmic expression of pYAP might occur if the Hippo pathway is preserved and there are further means of overcoming the inhibition *via* high-cell-density contact in ESCC. Nuclear YAP expression was related to tumor proliferation, which was estimated from the Ki-67 expression, whereas cytoplasmic expression of pYAP did not correlate with Ki-67 expression, although pYAP cytoplasmic expression was higher in ESCC than in normal esophageal epithelia (Tables I and II).

In conclusion, the results of this study indicate that increased nuclear expression of YAP in ESCC is an independent prognostic marker for shorter overall survival.

Table III. *Cox proportional odds ratio analysis for overall survival including as variables, Nuclear Yes-associated protein (YAP) expression and clinicopathological variables in esophageal squamous cell carcinoma.*

Factor	Odds ratio	95% Confidence interval	*p*-Value
Nuclear YAP expression			
Low	Reference value		
High	2.006	1.052-3.825	0.034
Cytoplasmic pYAP expression			
Low	Reference value		
High	0.957	0.515-1.779	0.890
Age, years			
<65	Reference value		
≥65	1.335	0.623-2.861	0.457
Gender			
Male	Reference value		
Female	0.000	0.000-7.990	0.969
Radiotherapy			
Not done	Reference value		
Done	0.743	0.403-1.371	0.342
Chemotherapy			
Not done	Reference value		
Done	0.793	0.396-1.585	0.511
Tumor stage			
I	Reference value		0.007
II	1.511	0.378-6.043	0.559
III	2.169	0.630-7.472	0.220
IV	11.663	2.348-57.933	0.003
Node stage			
0	Reference value		
1-3	2.031	0.965-4.278	0.062
Histological grade			
Well-differentiated	Reference value		0.564
Moderately differentiated	1.683	0.586-4.832	0.333
Poorly differentiated	1.924	0.559-6.625	0.300
Ki-67 (range 0-15)	0.929	0.827-1.042	0.208

Increased nuclear expression of YAP in ESCC correlates with tumor diameter, Ki-67 expression, histological grade (1-2 *versus* 3), pathological T category (I *versus* II-IV) and pathological TNM stage (I *versus* II-IV). These results suggest that nuclear YAP expression may act as a marker of tumor growth and be a prognostic marker for worse outcome for this disease. The finding that cytoplasmic expression of pYAP is higher than in normal esophageal epithelia implies that phosphorylation of YAP is activated because of the higher cellular density in ESCC than in normal esophageal epithelia and that pYAP is an inactivated form of YAP. Elucidation of the mechanisms underlying the accumulation of cytoplasmic pYAP in ESCC require further study.

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