

Immunopositivity of Beclin-1 and ATG5 as Indicators of Survival and Disease Recurrence in Oral Squamous Cell Carcinoma

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Abstract. Aim: To evaluate the expression and prognostic value of two autophagy-related (Atg) proteins, Beclin-1 and Atg5, in human oral squamous cell carcinoma (OSCC) and to correlate findings with clinical outcomes. Patients and Methods: Immunohistochemistry for Beclin-1 and Atg5 was assessed in tumor specimens from 90 patients with OSCC. Immunopositivity was semi-quantitatively scored and receiver-operating characteristic curve analysis was used to determine the cut-off positivity score. Results: 55 (61.1%) and 52 (57.8%) cases showed positive Beclin-1 and Atg5 staining, respectively. 40 tumors (44.4%) were positive for both Beclin-1 and Atg5 expression and 23 cases (25.6%) showed absence

of both proteins. Beclin-1 expression significantly correlated with tumor grade ($p=0.008$) and lymph node metastasis ($p=0.009$). The expression of Atg5 was associated with tumor grade ($p=0.016$), advanced clinical stage ($p<0.001$), large tumor size ($p=0.002$), and lymph node metastasis ($p<0.001$). A significant difference in 3-year OS ($p=0.050$) and TTR ($p=0.049$) between the patients with Beclin-1 expression and those not showing Beclin-1 expression was found whereas the difference did not reach a statistical significance for Atg5 expression. 3-year OS and TTR differed significantly between patients with dual expression and those with double-negative expression ($p=0.022$ and $p=0.026$, respectively). Conclusion: Dual expression of tumor Beclin-1 and Atg5 expression may be an adverse prognostic indicator for OSCC.

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Oral squamous cell carcinoma (OSCC), the most common malignancy of the oral cavity, representing a major public health issue in many parts of the world and is one of the leading causes of cancer-related death (1). Despite the fact that recent advances in therapeutic modalities have resulted in a decrease of overall death rate from cancer, the survival rate for patients with OSCC has remained virtually unchanged over the last three decades (2, 3). Regardless of

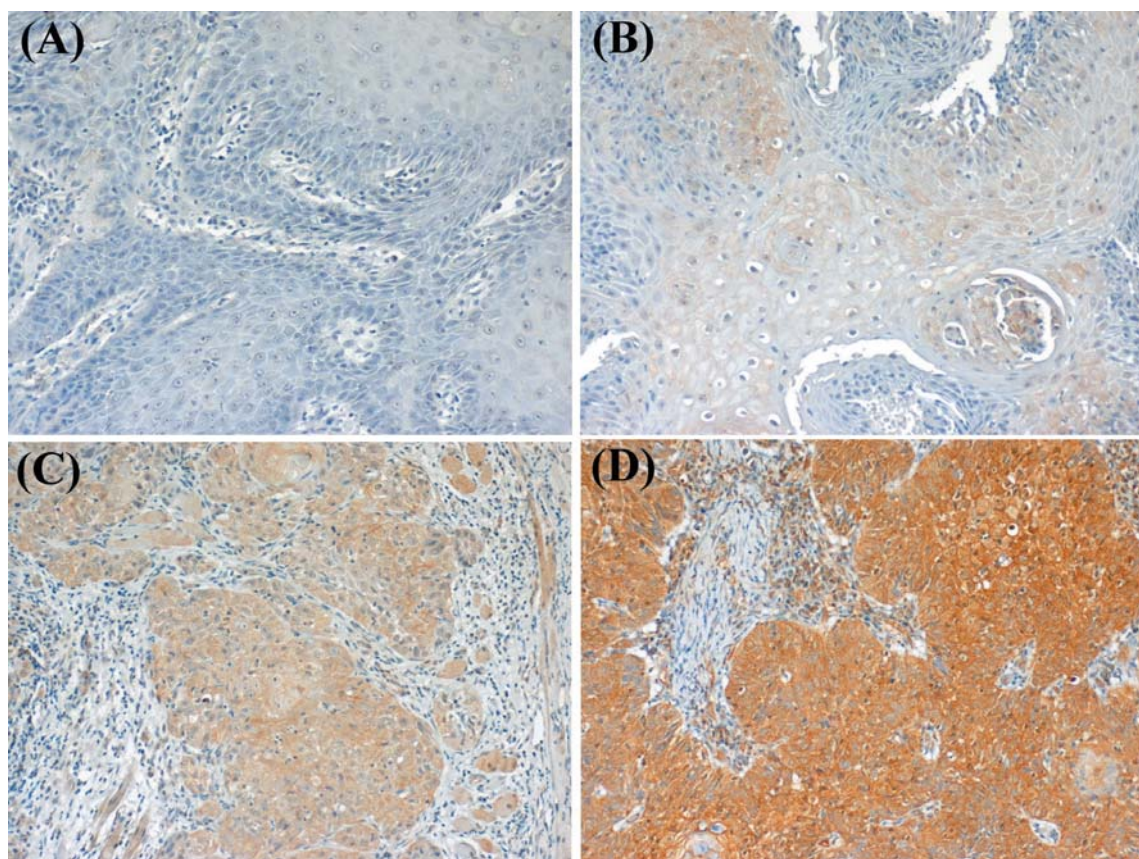


Figure 1. Representative immunostainings for Beclin-1 expression in tumor cells of oral squamous cell carcinoma (original magnification $\times 200$). Immunoreactivity for Beclin-1 was classified as (A) negative (IRS=0), (B) negative (IRS=2), (C) positive (IRS=4), and (D) positive (IRS=9).

the affected region in the oral cavity, OSCC is usually diagnosed in advanced stages. Early detection, in combination with more effective treatment approaches, have the potential to control for oral tumors and improve on patient outcome.

Autophagy is an intracellular bulk degradation system that plays various physiological roles such as development, differentiation and maintenance of cellular homeostasis (4, 5). Dysfunction of autophagy is related to severe diseases including myopathies, neurodegenerative diseases and cancer (5-7). This multi-step process is carried-out by a group of evolutionarily-conserved proteins called autophagy-related proteins (Atg) (6, 8). Beclin-1, the mammalian orthologue of the yeast Atg6, is a key mediator of autophagy. It interacts with several co-factors that regulate autophagy and its dysfunction has been implicated in many disorders including many types of human cancer (9). Induction of autophagy is initiated by the formation of double membrane structures called autophagosomes (8). An autophagosome encloses cargoes such as organelles and microbes and fuses with lysosomes which degrade these cargoes. The formation of

autophagosomes involves a ubiquitin-like conjugation system in which Atg5 is covalently-bound to Atg12 (10). Atg5, required for the covalent modification system, is essential for occurrence of autophagy (11).

Recent evidence suggests that autophagy may play an important role in the development and progression of OSCC (12). In the present study, we investigated the expression of two important proteins in the autophagy machinery, Beclin-1 and Atg5, and their correlation with clinical outcomes of patients with OSCC.

Materials and Methods

Tissue samples. Paraffin-embedded tissue samples from 90 primary OSCCs were collected from the archive of the Department of Pathology, Kaohsiung Medical University Hospital, Taiwan. All patients had previously received surgery as initial treatment between 2005 and 2009. The clinical and pathological data were obtained from the cancer registry and medical charts. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital and all enrolled patients provided their written informed consent.

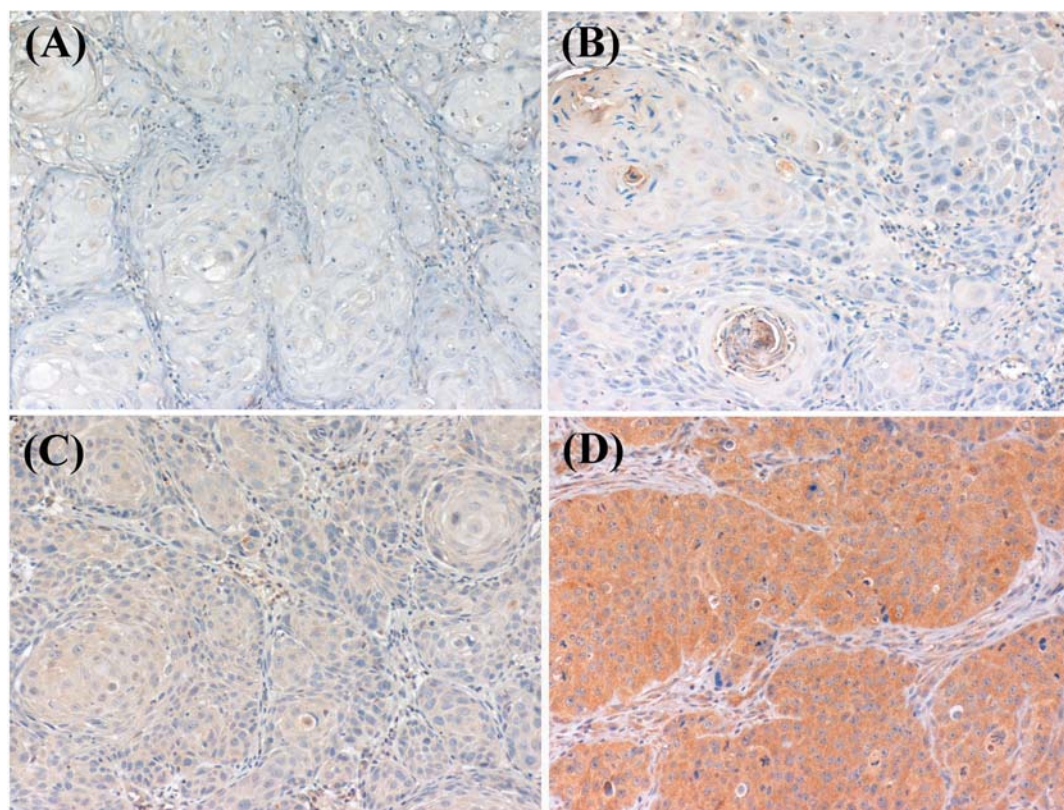


Figure 2. Representative immunostainings for Atg5 expression in tumor cells of oral squamous cell carcinoma (original magnification $\times 200$). Immunoreactivity for Atg5 was classified as (A) negative (IRS=0), (B) negative (IRS=2), (C) negative (IRS=3), and (D) positive (IRS=6).

Immunohistochemistry. Four-micrometer-thick paraffin-embedded sections were de-paraffinized in xylene and dehydrated through graded alcohols. Antigen retrieval was performed in 0.1 M citrate buffer (pH 6.0) at 121°C for 10 min. The slides were then incubated in 3% hydrogen peroxide at room temperature to quench endogenous peroxidase. Incubation with anti-Atg5 (1:400; Novus Biologicals, Littleton, CO, USA) or anti-Beclin-1 (1:75; Novus Biologicals,) was performed for 1 h at room temperature. The antigen-antibody complexes were visualized using the DAKO REAL Envision Detection System, Peroxidase/DAB, Rabbit/Mouse (DAKO, Denmark), followed by hematoxylin counterstaining and mounting. Normal ductal breast tissues and breast cancer tissues were used as positive controls for immunohistochemistry. Negative controls were obtained by replacing the primary antibody with non-immune serum.

The immunoreactivity of Atg5 and Beclin-1 was evaluated on the basis of the number of cells (proportion) that were stained and the staining intensity, and was performed by pathologists blinded to clinical data. The intensity of immunoreactivity was classified as negative: 0, weak: 1, moderate: 2, strong: 3. Regarding scoring proportions the slides were deemed as follows: 0, <1% tumor cells showing immunoreactivity; score 1, 1-10% showing immunoreactivity; score 2, 11-50% showing immunoreactivity; score 3, >50% showing immunoreactivity. The immunoreactivity score (IRS), ranging from 0 to 9, was calculated by multiplying the staining intensity by the proportion score.

Table I. Pattern of expression and correlation between Beclin-1 and Atg5 expression in oral squamous cell carcinoma.

	ATG5			Kappa value
	Negative	Positive	Total	
Beclin-1				
Negative	23 (25.6%)	12 (13.3%)	35 (38.9%)	0.38
Positive	15 (16.7%)	40 (44.4%)	55 (61.1%)	
Total	38 (42.2%)	52 (57.8%)	100 (100.0%)	

Statistical analysis. Determination of the expression cut-off value predicting survival was made using the receiver operating characteristics (ROC) curve. Association between expression levels and clinicopathological characteristics of the OSCC patients were analyzed by the Chi-square test. Kaplan-Meier analysis was used to estimate overall survival (OS) and time-to-recurrence (TTR). OS was calculated from the time of initial diagnosis until death. TTR was calculated from the time of diagnosis to disease recurrence. Patients still alive or without evidence of recurrence were censored at last follow-up. A log-rank test was used to determine statistical significance. A p -value of <0.05 was considered to denote statistical significance.

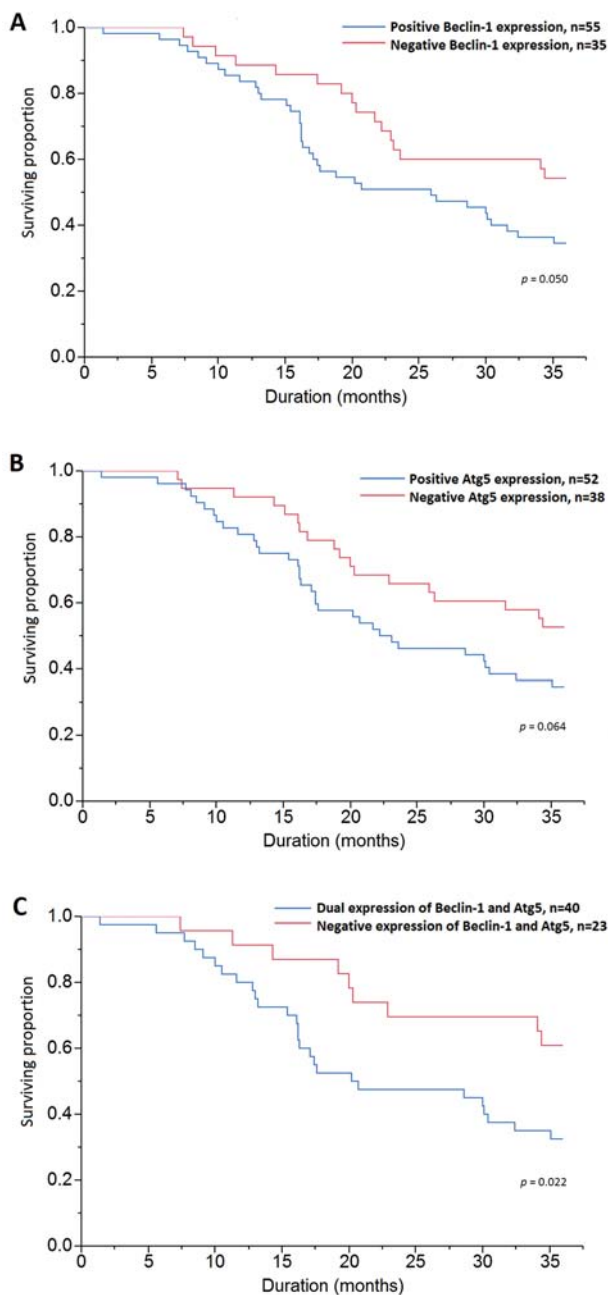


Figure 3. Kaplan-Meier estimation of the cumulative proportion of overall survival according to Beclin-1 expression (A), Atg5 expression (B), and dual expression of Beclin-1 and Atg5 (C) in patients with oral squamous cell carcinoma.

Results

Cytoplasmic Beclin-1 and Atg5 expression at varying levels were found in the studied OSCC samples. According to the ROC curves for survival analyzed in our study, threshold

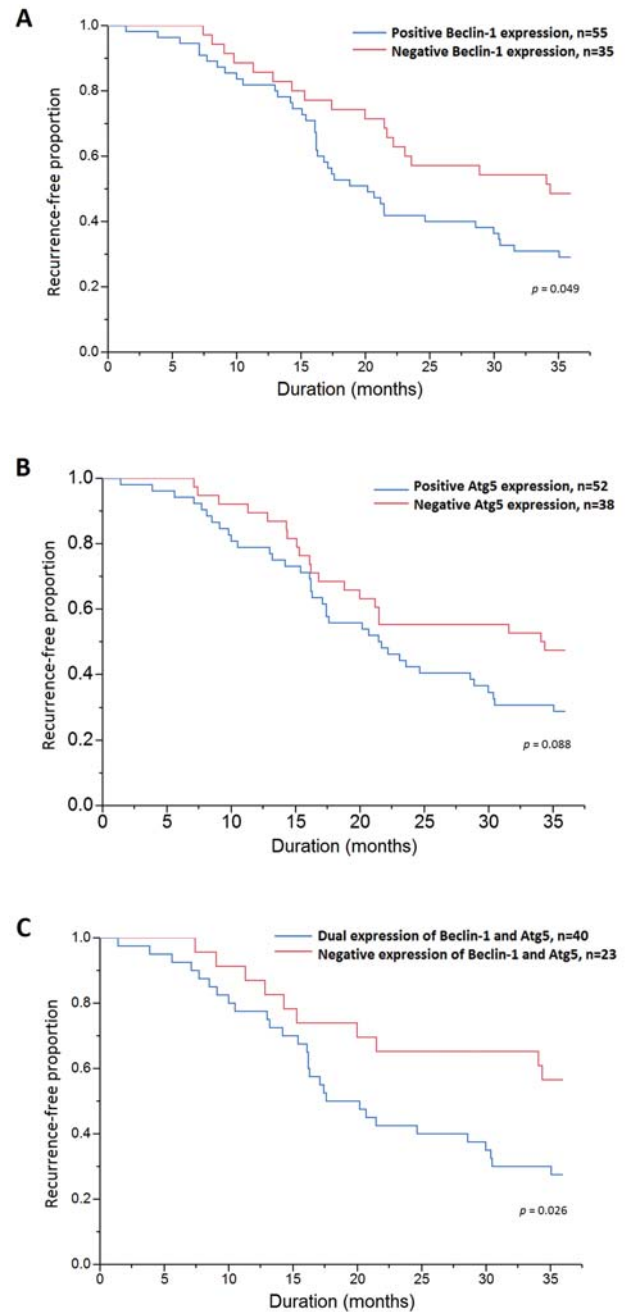


Figure 4. Kaplan-Meier estimation of the cumulative proportion of no tumor recurrence according to Beclin-1 expression (A), Atg5 expression (B), and dual expression of Beclin-1 and Atg5 (C) in patients with oral squamous cell carcinoma.

values of 4 for Beclin-1 and 6 for Atg5 were the closest to the point with both maximum sensitivity and specificity, and were thereby selected as cut-off IRS values. Therefore, OSCC cases with a Beclin-1 IRS value ≥ 4 were regarded as Beclin-1-positive (Figure 1). Likewise, immunopositivity of

Table II. Analysis of Beclin-1 and Atg5 expression in oral squamous cell carcinoma: correlation with clinicopathological characteristics.

	Beclin-1 expression				Atg5 expression			
	Negative (n=35)	Positive (n=55)	n	p-Value	Negative (n=38)	Positive (n=52)	n	p-Value
Age, years, mean (SD)	57.4 (11.6)	53.5 (10.7)	90	0.118	55.1 (11.2)	55.1 (11.2)	90	0.950
Tumor grade								
I	25 (75.8%)	27 (55.1%)	52	0.008	26 (74.3%)	26 (55.3%)	52	0.016
II+III	8 (24.2%)	22 (44.9%)	30		9 (25.7%)	21 (44.7%)	30	
Tumor stage								
I	9 (25.7%)	7 (12.7%)	16	0.212	12 (31.6%)	4 (7.7%)	16	<0.001
II	10 (28.6%)	13 (23.6%)	23		17 (44.7%)	6 (11.5%)	23	
III	9 (25.7%)	14 (25.5%)	23		6 (15.8%)	17 (32.7%)	23	
IV	7 (20.0%)	21 (38.2%)	28		3 (7.9%)	25 (48.1%)	28	
Tumor size								
≤20 mm	9 (25.7%)	9 (16.4%)	18	0.524	12 (31.6%)	6 (11.5%)	18	0.002
>20mm, ≤40 mm	13 (37.1%)	21 (38.2%)	34		18 (47.4%)	16 (30.8%)	34	
>40mm	13 (37.1%)	25 (45.5%)	38		8 (21.1%)	30 (57.7%)	38	
Lymph node metastasis								
-	22 (62.9%)	19 (34.5%)	41	0.009	27 (71.1%)	14 (26.9%)	41	<0.001
+	13 (37.1%)	36 (65.5%)	49		11 (28.9%)	38 (73.1%)	49	

Atg5 was defined as an Atg5 IRS value ≥ 6 (Figure 2). Table I depicts the pattern of the expression for Beclin-1 and Atg5 in the current cohort of OSCC patients. Out of 90 OSCC samples, 55 (61.1%) and 52 (57.8%) showed positive Beclin-1 and Atg5 staining, respectively. Chi-square trend analysis revealed that Beclin-1 expression correlated with tumor grade ($p=0.008$) and lymph node metastasis ($p=0.009$) (Table II). The expression of Atg5 was associated with tumor grade ($p=0.016$), advanced clinical stage ($p<0.001$), large tumor size ($p=0.002$), and lymph node metastasis ($p<0.001$) (Table II). According to the results of the Kaplan-Meier analysis, there was a significant difference in 3-year OS ($p=0.050$) and TTR ($p=0.049$) between patients with Beclin-1 expression and those not showing Beclin-1 expression (Figure 3A and 4A). Trends not reaching statistical significance were shown between Atg5 expression and OS ($p=0.064$) and TTR ($p=0.088$) (Figure 3B and 4B). Of note, 40 OSCC cases (44.4%) revealed a dual expression of Beclin-1 and Atg5 and 23 cases (25.6%) showed absence of Beclin-1 and Atg5 expression. Expression of Beclin-1 was positively correlated with Atg5 expression in OSCC tumors (Kappa=0.38; Table I). Three-year OS and TTR differed most significantly between patients with dual expression and those with double-negative expression ($p=0.022$ and $p=0.026$, respectively; Figure 3C and 4C).

Discussion

Although dysregulation of autophagy has been reported in a wide variety of cancers, the role of autophagy which can both promote cell death and survival is not yet well-understood in

human cancers, and contradictory results of the role of autophagy on the oncogenesis of OSCC have been reported (12-15). Atg proteins are required for the biogenesis of the autophagosome and function in a hierarchical manner during the different stages of autophagosome formation. In the present study, we investigated the expression of two Atg proteins, Beclin-1 and Atg5, in OSCC primary tissues through immunohistochemistry. Beclin-1 and Atg5 were expressed in 55 (61.1%) and 52 (57.8%) of the 90 cases examined, respectively. Cytoplasmic expression of Beclin-1 correlated with tumor grade ($p=0.008$) and the presence of lymph node metastasis ($p=0.009$). On the other hand, Atg5 cytoplasmic expression was statistically associated with tumor grade ($p=0.016$), advanced clinical stage ($p<0.001$), large tumor volume ($p=0.002$), and lymph node metastasis ($p<0.001$). Kaplan-Meier analysis indicated that the expression of Beclin-1 can serve as a prognostic role for both OS and disease recurrence ($p=0.05$ and $p=0.049$, respectively). OS and TTR were shorter in patients with cytoplasmic Atg5 expression when compared to patients without expression but the difference did not reach statistical significance. Furthermore, we found that expression of Beclin-1 was significantly positively correlated with the expression of Atg5 in OSCC, and patients with dual expression of both Beclin-1 and Atg5 had a worse prognosis than those with dual-negative expression. Three-year OS and TTR differed most significantly between patients with dual expression and those with dual-negative expression ($p=0.022$ and $p=0.026$, respectively).

Altered expression of several autophagy markers has been reported in human carcinomas and it appeared that both reduced expression and overexpression may occur (16). The

findings of the current study which were based on a larger case series and different study design do not support the results of a previous study by Kapoor *et al.* reporting that mRNA levels of *Beclin-1* were down-regulated in tumor tissues of 10 OSCC patients (13).

This is the first study to show the usefulness of immunohistochemical staining and scoring of two Atg proteins in selecting high-risk OSCC populations. Moreover, we hypothesized that OSCCs have a high degree of autophagy activity and that antibodies against critical proteins involved in autophagy can be used to characterize the dysregulation of autophagy. Our study suggests that the immunohistochemical assessment of Beclin-1 and Atg5 may represent a valid tool in clinical practice, due to the general availability of formalin-fixed samples, for identifying subgroup of OSCC patients with poor clinical outcome.

Conflicts of Interests

The Authors declare that they have no competing interests.

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