• LIVER CANCER •

Expression and altered subcellular localization of the cyclin-dependent kinase inhibitor p27^{Kip1} in hepatocellular carcinoma

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Abstract

AIM: To investigate p27 expression in hepatocellular carcinoma (HCC), adjacent nontumoral and normal liver tissues, and to verify whether the subcellular localization of p27 was altered in HCC.

METHODS: The level of p27 in tumoral, nontumoral, and normal liver tissues were assessed by immunohistochemical (IHC) analysis. Parallel immunostaining was done for proliferating cell nuclear antigen (PCNA) to evaluate cell proliferation.

RESULTS: The labeling index (LI) of p27 in tumoral lesions was significantly lower than that in adjacent nontumoral lesions ($t=2.444$, $P=0.017$) and normal controls ($t=2.268$, $P=0.029$). The LI of p27 significantly decreased in patients with massive type ($t=2.227$, $P=0.037$) and infiltration ($t=2.197$, $P=0.036$). The prognosis of patients with higher p27 LI was longer than that of patients with lower p27 LI ($P=0.0247$, log-rank test). The LI of PCNA was significantly higher in HCC than that in adjacent nontumoral lesions ($t=2.092$, $P=0.041$) and normal controls ($t=3.533$, $P=0.002$). There was no significant correlation between p27 expression and cell proliferation in tumor samples. The level of p27 in the cytoplasmic fraction was higher in tumoral and nontumoral liver tissues, and was associated with clinical stage ($t=2.520$, $P=0.029$) and the degree of invasion ($t=2.640$, $P=0.019$). Survival analysis showed that p27 was an independent prognosis marker for HCC patients.

CONCLUSION: These results suggest that p27 underexpressing in patients with HCC is closely associated with infiltration, metastasis, and prognosis. Alterations in the subcellular localization of p27 protein may occur early during hepatocarcinogenesis.

INTRODUCTION

Abnormalities in various regulators of cell cycle frequently occur in human cancers[1-4]. Cell cycle is tightly controlled by the regulators, including cyclins, cyclin-dependent kinases (CDKs), and their inhibitors. Cyclin-dependent kinase inhibitors (CKIs) are potent negative regulators of cell cycle, and have two families on the basis of sequence homology. The inhibitors of CDK4 (INK4) family, including p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4}, specifically binds to CDK4 and CDK6, and inhibits cyclin D binding. The Cip/Kip family, including p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, binds to and inhibits cyclin-bound CDKs[5]. Among CKIs, p27 is thought to have crucial[6] and important roles in a variety of fundamental cellular processes[7-10]. The underexpression of p27 assessed by IHC analysis has been associated with more severe tumor grade and a negative prognostic marker in different carcinomas[11-16].

Furthermore, studies showed that reduced p27 expression was correlated with advanced tumor stages and poorer disease-free survival in patients with HCC at a variety of evolutionary stages[17-20]. However, most precious investigations are based on immunostaining of tumoral liver samples without comparing with surrounding nontumoral liver, which should be the best control tissue to be used for comparison.

In addition, p27 functions at the nuclear level by binding to and inhibiting cyclin/CDK complexes. It was recently reported that cytoplasmic displacement was an alternative way to inhibit p27 activity and might also play a role in tumor development[21]. Indeed, positive immunostaining of cytoplasmic p27 has been previously reported in various human cancers[22-25]. To address this hypothesis, the expression of p27 in tumoral, adjacent nontumoral, and normal liver tissues was evaluated by IHC analysis in patients with HCC, and the subcellular distribution of p27 was also investigated in this study.

MATERIALS AND METHODS

Patients and tumor samples

From January 2000 to December 2001, 32 liver samples were collected from patients who had HCC and underwent surgery in our institution. All liver tumors were histologically diagnosed. Tumor gross type and stage were diagnosed using Eggel' s classification and Union International Contre Cancer (UICC) criteria. Tumor cellular differentiation was identified by Edmondson' s classification. No patient received pre-operative chemotherapy or chemoembolization. The clinicopathologic characteristics of 32 patients with HCC are listed in Table 1. Normal livers were prepared from 10 patients who were died from the accident as the controls.

After curative surgery, all patients were followed up every 3 mo till death. They were followed from 2 to 32 mo (mean 15.2 mo). Actuarial survival was measured from the day of surgical operation to that of death or last follow-up.

Immunohistochemical study

Liver samples were routinely fixed in 40 g/L formaldehyde solution and embedded in paraffin. After slicing into 4 μm-thick sections, IHC analysis was performed using Dako Elivision™ plus two-step System. In brief, the sections were dewaxed in xylene, and rinsed in alcohol and graded alcohol/water mixtures. Then, 30 mL/L hydrogen peroxide was applied to block

endogenous peroxidase activity. The sections were subsequently treated in a microwave oven twice for 5 min in citrate buffer (pH 6.0) at high power. After blocking with goat serum, the mouse monoclonal antibodies against p27 (ZM-0340) and PCNA (ZM-0213) (Zymed Biotechnology, Zymed, CA) were applied on the slides at the dilution of 1:120 and 1:50, respectively. After rising, staining was performed by the Elivision™ plus two-step System (kit-9902, Dako, Carpinteria, CA). The color was developed by reacting with 3,3-diaminobenzidine. Slides were then counterstained with hematoxylin, dehydrated, cleared, and mounted. Negative controls were performed by replacing the primary antibody with nonimmune mouse serum or PBS (Figure 1). A human breast cancer specimen and the reactive tonsil were used as positive controls for p27 (Figure 2) and PCNA, respectively.

Table 1 Clinicopathologic characteristics of 32 Patients

Clinicopathologic parameters	Number	%
Age		
$\leqslant$45 years	14	43.8
>45 years	18	56.2
Gender		
Male	26	81.2
Female	6	18.8
Gross type		
Massive	15	46.9
Nodular	17	53.1
Tumor size		
$\leqslant$5 cm	5	15.6
>5 cm	27	84.4
Cellular differentiation		
I	4	12.5
II	17	53.1
III	5	15.6
IV	6	18.8
Degree of invasion		
T_1+T_2	12	37.5
T_3+T_4	20	62.5
Stage		
I+II	10	31.2
III+IV	22	68.8
Lymph node metastasis		
Present	4	87.5
Absent	28	12.5
Portal invasion		
With	3	9.4
Without	29	90.6
Infiltration		
Present	12	37.5
Absent	20	62.5

Immunostaining evaluation
Slides were mounted independently by 2 investigators without notifying any clinical or pathological information. The positive cells for p27 and PCNA were measured by counting at least 1 000 HCC cells from at least 5 randomly selected fields under the microscope. Then, LI was calculated as a percentage for each protein.

Statistical analysis
Values were expressed as mean±SD, The Student's *t* test was performed to analyze the relationship between the expression of the proteins and various clinicopathologic parameters. The

cumulative survival rate was computed and actuarial survival curves were constructed by the Kaplan-Meier method. The cumulative survival rate was compared between groups using the log-rank test. Relevant prognostic factors were identified by univariate and multivariate Cox proportional hazard regression analyses. Tests were considered significant when the *P* values were <0.05.
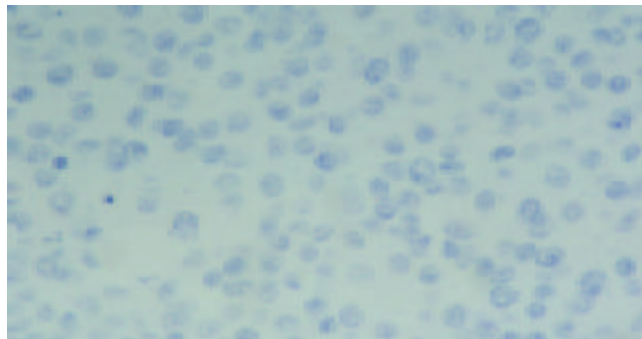

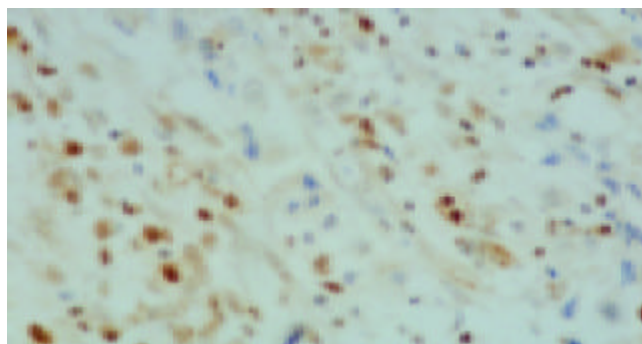
Figure 1 Negative control of p27 (×400).

Figure 2 Positive control of p27 (×400).

RESULTS

Reduced expression of p27 in HCC
The mean LI of p27 in HCC was 27.7±19.9%, which was lower than that in adjacent nontumoral lesions (42.3±21.4%, *t*=2.444, *P*=0.017) and that in normal controls (39.1±17.5%, *t*=2.268, *P*=0.029). Furthermore, the LI of p27 in adjacent nontumoral lesions was not significantly different from that in normal controls (Figure 3). Table 2 shows the relationship between p27 expression and clinicopathologic characteristics. The LI of p27 significantly decreased in patients with massive type (*t*=2.227, *P*=0.037) and infiltration (*t*=2.197, *P*=0.036).

Proliferating activity in HCC
The mean LI of PCNA was 49.5±14.2% in HCC, which was higher than that in adjacent nontumoral lesions (42.8±11.2%, *t*=2.092, *P*=0.041) and normal controls (36.2±8.9%, *t*=3.533, *P*=0.002). The LI of PCNA in adjacent nontumoral lesions was not significantly different from that in normal controls (Figure 4). The LI of PCNA significantly increased in cases with poor differentiation (*t*=2.259, *P*= 0.031, Table 2). The patients were divided into low p27 and high p27 groups with the cut-off value of the median LI. The LI of PCNA was 51.2±15.6% and 48.1±13.0% in low p27 and high p27 groups, respectively. There was no significant difference between these two groups (*t*=0.578, *P*=0.568).

Altered subcellular localization of p27 in HCC
The cytoplasmic expression of p27 was found in the HCC,

adjacent nontumoral, and normal liver tissues. Therefore, the cytoplasmic displacement of p27 might not be a specific phenomenon of tumor cells. However, the cytoplasmic sequestration of p27 was more frequent in HCC and adjacent nontumoral lesions (Figure 3).

The mean LI of cytoplamic p27 was 31.0±12.6% in adjacent nontumoral lesions, which was higher than that in HCC (21.6±18.2%, t=2.378, P=0.021) and normal controls (13.9±5.6%, t=5.994, P<0.001). The LI of cytoplamic p27 in HCC was higher than that in normal controls (t=2.106, P=0.042).

Whereas, the nuclear LI in normal controls (31.3±12.6%) was higher than that in HCC (12.5±9.7%, t=4.968, P<0.001) and nontumoral lesions (16.5±10.4%, t=3.731, P=0.001). Altered subcellular localization of p27 was correlated with clinical stage (t=2.520, P=0.029) and the degree of invasion (t=2.640, P=0.019) (Table 2).

Expression of p27 and PCNA and survival
The survival rate of 6, 12, and 24 mo was 71.9%, 47.8%, and 25.1%, respectively. The patients were divided into 2 groups according
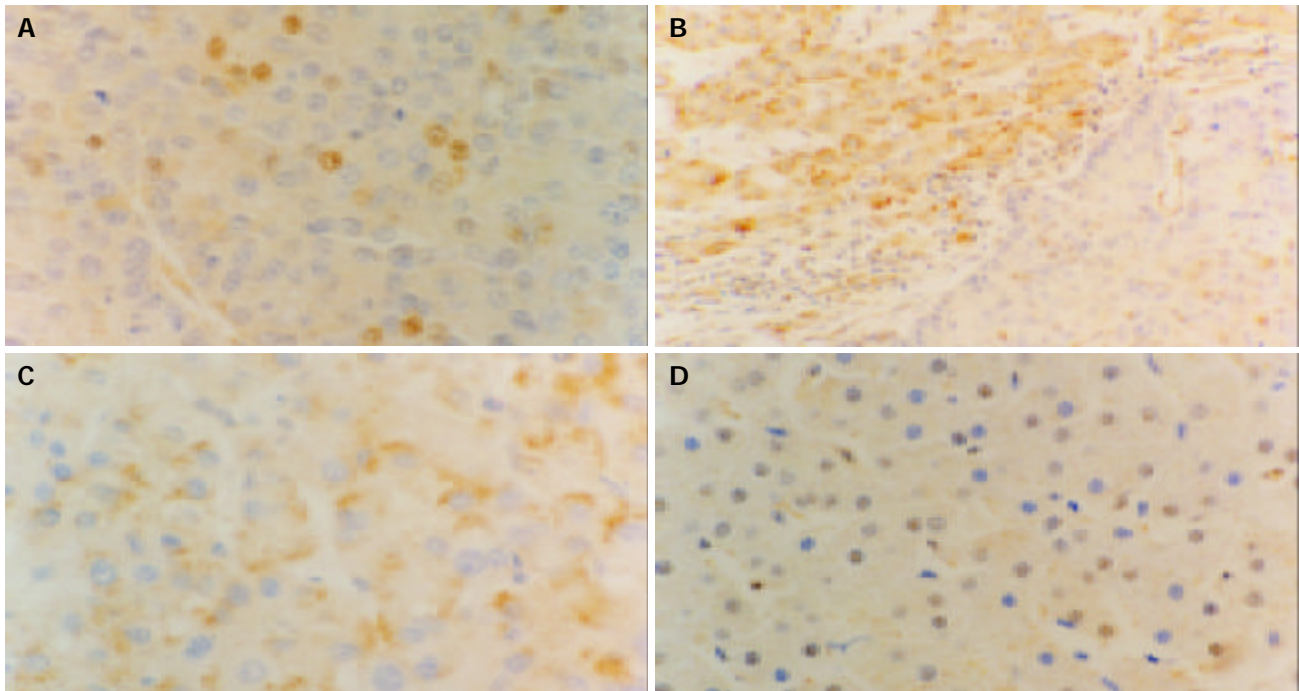

Figure 3 Immunohistochemical staining of p27 in different liver tissues(Elivision), A: p27 expression is decreased in HCC ×400, B: Decreased p27 expression in HCC (right), the p27 expression is mostly located in the cytoplasm in nontumoral liver tissues (left) ×200, C: Some tumor cells show cytoplasmic staining of p27 ×400, D: p27 staining in normal controls is mostly located in nuclear ×400.
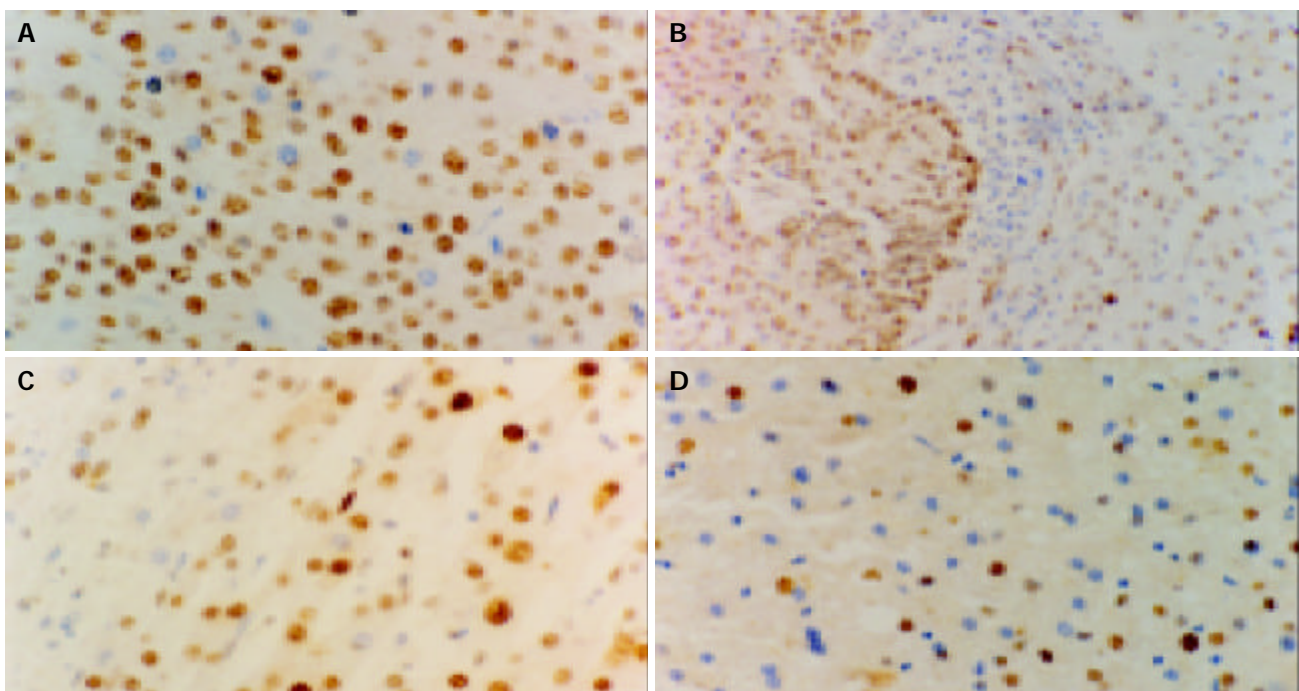

Figure 4 Expression of PCNA in different liver tissues(Elivision), A: Expression of PCNA in HCC ×400, B: Expression of PCNA in HCC(left) and in nontumoral liver tissues(right) ×200, C: Expression of PCNA in nontumoral liver tissues ×400, D: in normal controls ×400.

Table 2 Relationship among p27, PCNA, and cytoplasmic p27staining with clinicopathologic features

Parameters	p27 LI (%)	P	PCNA LI (%)	P	Cytoplasmic p27 LI (%)	P
Gross type						
Massive	20.5±13.1	0.037	46.4±13.4	0.249	25.9±18.9	0.221
Nodular	35.7±23.4		52.3±14.7		17.9±17.3	
Tumor size						
≤5 cm	23.8±14.4	0.645	38.1±10.7	0.047	27.0±24.1	0.481
>5 cm	28.4±20.9		51.7±13.9		20.6±17.4	
Cellular differentiation						
Well or moderate	29.7±21.4	0.438	45.7±13.8	0.031	23.2±18.0	0.498
Poor	23.8±17.1		56.9±12.6		18.6±19.1	
Degree of invasion						
T_1+T_2	32.6±23.3	0.285	51.1±14.7	0.64	33.0±22.3	0.019
T_3+T_4	24.7±17.5		48.6±14.4		14.8±11.1	
Stage						
I+II	33.6±25.6	0.261	48.0±12.9	0.688	35.3±23.9	0.029
III+IV	24.9±16.7		50.2±15.0		15.4±10.7	
Infiltration						
Present	18.3±18.8	0.036	44.4±12.3	0.117	19.0±18.3	0.536
Absent	33.3±18.7		52.6±14.7		23.2±18.5	
Lymphocyte metastasis						
With	25.5±2.6	0.565	56.0±14.9	0.339	16.0±10.6	0.518
Without	27.9±21.3		48.6±14.2		22.4±19.1	
Portal invasion						
Present	14.3±12.7	0.229	58.3±12.0	0.267	13.0±11.8	0.398
Absent	29.1±20.1		48.6±14.3		22.5±18.7	

to the median LI of p27, cytoplasmic p27, and PCNA. The survival analysis was performed on 32 patients and took into account for the following variables: age, gender, gross type, tumor size, UICC tumor stage, Edmondson's grade, portal invasion, lymph node metastasis, depth of invasion, infiltration, p27 LI, cytoplasmic p27 LI, and PCNA LI. In univariate analysis, a significant correlation with short survival was found only for present portal invasion ($P=0.021$) and low p27 expression ($P=0.033$). The prognosis of patients with higher p27 LI was longer than that of patients with lower p27 LI ($P=0.0247$, log-rank test, Figure 5). Multivariate analysis was performed by Cox proportional hazard regression model, age ≥45 years ($P=0.016$) and low p27 expression ($P=0.009$) were significantly associated with shorter survival. In addition, there was a trend of present lymphocyte metastasis ($P=0.05$) and advanced stage ($P=0.08$) correlated with poor prognosis (Table 3). For gross type, tumor size, Edmondson grade, depth of invasion, infiltration, cytoplasmic p27 LI, and PCNA LI, no significant correlations with overall survival were found.
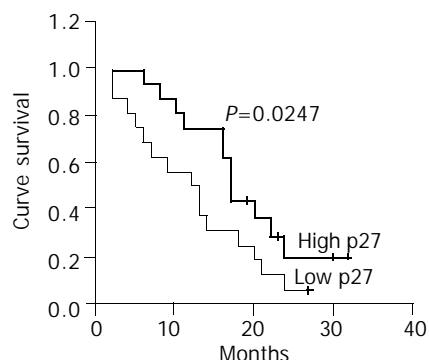
Figure 5 Kaplan-Meier curve for actuarial survival of patients in low p27 and high p27 groups.

Table 3 Univariate and multivariate analyses of individual parameters

Variables	P Values	
	Univariate	Multivariate
Age	0.150	0.016
Tumor size	0.513	0.678
Carcinoma differentiation	0.415	0.628
Stage	0.200	0.080
Lymphocyte metastasis	0.821	0.050
Infiltration	0.317	0.849
Portal invasion	0.021	0.619
p27	0.033	0.009
Cytoplasmic p27	0.114	0.624
PCNA	0.915	0.282

DISCUSSION

Hepatocellular carcinoma is one of the most common malignant tumors in China, and the incidence of HCC has increased in recent years[26]. Cell proliferating activity is one of the prominent parameters for evaluating the biological aggressiveness of tumors. The study in cell proliferating status of HCC is very important for evaluating the biological aggressiveness[27,28], because distant and lymph node metastasis are not very common.

The protein p27 is an important member of CKI family. It regulates G1 progression by binding to and inhibiting cyclin/CDKs[29,30]. Loss or reduced expression of p27 has been found in a variety of human cancers and associated with more aggressive tumor behavior. The present study showed that p27 expression significantly decreased in HCC. The finding of p27 underexpression in HCC was similar to the previous reports[31]. This study showed that the expression of p27 in

HCC was associated with gross type and direct liver invasion. Univariate analysis showed that portal invasion and p27 LI were related to survival in this study. In multivariate analysis, p27 expression was the strongest predictor of survival for HCC patients, which was independent of the other variables. Similar findings have also been observed in other cancers[32-34]. It is therefore suggested that p27 should work as a negative regulator during hepatocarcinogenesis and may exert a useful prognostic marker. Interestingly, as reported by Milde-Langosch[35], there was no correlation between p27 expression and proliferating level, indicating that reduced expression of p27 did not merely reflect high proliferating activity during tumor progression.

To our knowledge, this was the first study to report the altered subcellular localization of p27 in HCC. The protein p27 can bind to and inhibit the active cyclin/CDK complexes in the nucleus, so the cytoplasmic displacement may play an important role in inactivating this protein in tumor cells and in contributing to tumor development. Indeed, cytoplasmic localization of p27 immunostaining has been reported in various types of human cancers. In our study, we found that the cytoplasmic localization of p27 was more frequent in HCC and surrounding nontumoral livers. Furthermore, the cytoplasmic staining for p27 was more frequently accompanied with nuclear staining in normal controls. However, the increase in the cytoplasmic staining for p27 was often observed in the absence of a concomitant increase in the nuclear staining and was sometimes associated with a decrease in the nuclear staining. Some tumors expressed an increased level of p27 mainly because of an increase in the cytoplasmic level of this protein. The increase in the amount of cytoplasmic p27 was more frequent in early stage (I and II) tumors. Altered subcellular localization of p27 was also reported in Barrett's associated adenocarcinoma and colon cancer[22,23]. In agreement with our results, cytoplasmic localization of p27 was an early event during carcinogenesis.

Although the mechanism responsible for the abnormal subcellular localization was not known, it may be due to loss of the tuberous sclerosis complex gene-2 (TSC2), the HER/Grb2/MAPK pathway which leads to nuclear export of p27[36], overexpression of cyclin D3 which contributes to retaining p27 in the cytoplasm[37], or PKB/Akt which phosphorylates p27 to impair its nuclear import[38-40].

In conclusion, our results suggest that underexpression of p27 can contribute to the development of HCC. Cytoplasmic displacement is an alternative mechanism of inactivating p27 that acts early during hepatocarcinogenesis. Furthmore, studies on a large number of cases will reveal the significance of p27 subcellular localization in hepatocarcinogenesis and its relationship with clinicopathologic and prognostic parameters.

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