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Bag-3 expression is involved in pathogenesis and progression of colorectal carcinomas

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Summary. Bcl-2-associated athanogene 3 (Bag-3) belongs to a member of the Hsc70 binding co-chaperone Bag-family proteins and has critical roles in protein homeostasis, cell survival, actin organization, cell adhesion, cell motility and tumor metastasis. To clarify the role of *Bag-3* in colorectal carcinogenesis and subsequent development, its expression was examined by immunohistochemistry (IHC) and *in situ* hybridization (ISH) on tissue microarrays containing colorectal carcinomas, adenomas, non-neoplastic mucosa (NNM) and metastatic carcinomas in lymph node and liver. Colorectal carcinoma tissue and cell lines were studied for Bag-3 expression by RT-PCR, Western blot and immunofluorescence. The results demonstrated that Bag3 was distinctly expressed in Colo201, Colo205, DLD-1, HCT-15, HCT-116, HT-29, KM-12, SW480, SW620, and WiDr at both mRNA and protein levels. Carcinoma showed stronger Bag-3 expression than adjacent NNM by IHC and Western blot ($P<0.05$), while its mRNA had the opposite by real-time PCR and ISH ($P<0.05$). Metastatic carcinoma more frequently expressed *Bag-3* mRNA in lymph node and liver than in primary carcinoma ($P<0.05$). Immunohistochemically, Bag-3 expression was seen to gradually decrease from carcinoma, adenoma to NNM ($P<0.05$). There was a positive correlation between Bag-3 expression and TNM staging and GRP94 expression ($P<0.05$), but no relationship to patient age or sex, tumor size, depth of invasion, lymphatic or venous invasion, lymph node metastasis, differentiation or prognosis of colorectal carcinomas ($P>0.05$). Our study indicated that aberrant Bag-3 expression might be involved in colorectal adenoma-adenocarcinoma sequence and subsequent progression.

Key words: Colorectal carcinoma, Bag-3, Patho-biological behavior, Prognosis

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Introduction

Colorectal cancer is one of the most common cancers in the world, accounting for nearly 10% of new cases of all cancer. Japan has experienced a marked increase in the incidence of colorectal cancer, and has recently been listed in the group of countries with the world's highest incidence rates (Boyle and Leon, 2002; American Cancer Society, 2005; Yoshida et al., 2007). Although pathological and genetic observations demonstrated that colorectal adenoma precedes the majority of adenocarcinoma, the molecular mechanisms underlying colorectal carcinogenesis are still poorly understood.

Bcl-2-associated athanogene 3 (Bag-3, also known as Bis or CAIR-1), a member of the Hsc70 binding co-chaperone Bag-family proteins, has critical roles in protein homeostasis, cell survival, actin organization, cell adhesion, cell motility and tumor metastasis. The human *Bag-3* is localized in chromosome 10q25.2-q26.2 and its 2608-nucleotide cDNA encodes the 575- amino-acid product with the molecular weight of 61kDa (Lee et

al., 1999). Bag-3 is a cytoplasmatic protein, particularly concentrated in the rough endoplasmic reticulum, while a doublet form or a nuclear localization can be observed in some cell types and/or following cell exposure to stressors. Bag-3 protein contains a WW domain and a proline-rich region with SH3-binding motifs, suggesting that it may interact with proteins relevant to signal transduction. Bag-3 overexpression in MDA435 human breast cancer cells results in a significant decrease in migration and adhesion to matrix molecules that is reversed upon deletion of Bag-3 proline-rich domain (Kassis et al., 2006), while the PPDY motif at the C-terminus of guanine nucleotide exchange factor 2 (PDZGEF2) binds to the WW domain of Bag-3, whose deletion results in the loss of cell adhesion and motility activity (Iwasaki et al., 2007).

Through the Bag domain, Bag-3 protein binds to the ATPase domain of Hsc70 and regulates its chaperone activity in a Hip-modulated manner. Through its PXXP region, Bag-3 binds to the SH3 domain of PLC-Á and forms an epidermal growth factor-regulated ternary complex. Its proline-rich repeat appears to be involved in regulating cell adhesion and migration through an indirect effect on focal adhesion kinase (Rosati et al., 2011). Bag-3 overexpression in tumors promotes survival by altering interaction between HSP70 and IKKgamma, increasing availability of IKKgamma and protecting it from proteasome-dependent degradation in the NF-kappaB pathway (Ammirante et al., 2010). Targeted suppression of Bag-3 results in dysregulation of cell-cycle progression, most notably at S and G2 phases, which corroborates the decreased level of cyclin B1 expression (Gentilella and Khalili, 2009). Bag-3 can initiate macroautophagy and participate in protein quality control via co-operation with small heat-shock protein HspB8 and the IPV (Ile-Pro-Val) motif in Bag-3 under the activation of the eIF2alpha signaling pathway (Carra, 2009; Fuchs et al., 2009). Bag-3 overexpression protected ubiquitinated clients, such as Akt, from proteasomal degradation and conferred cytoprotection against heat shock (Virador et al., 2009).

Bag-3 knockout mice developed normally, but deteriorated postnatally with stunted growth and fulminant myopathy characterized by noninflammatory myofibrillar degeneration with apoptotic features (Homma et al., 2006). In prostate carcinomas, Bag-3 showed a progressive decrease of the expression level from well- to low poorly-differentiated carcinoma, coupled with the loss of polarization of the signal in metastasizing cases (Staibano et al., 2010). Bag-3 protein is highly expressed in many human epithelial cancer cell lines, and is closely linked to tumor invasion and metastasis (Iwasaki et al., 2007). As to the roles of *Bag-3* expression in colorectal carcinogenesis and subsequent progression, we examined the expression of *Bag-3* mRNA and protein in colorectal non- neoplastic mucosa, adenoma, adenocarcinoma, and compared it with clinicopathological parameters and survival time of carcinomas.

Materials and methods

Cell lines and culture

Colorectal carcinoma cell lines were kindly presented by Prof. Sugiyama, Department of Gastroenterology, Graduate School of Medical and Pharmaceutical Sciences, University of Toyama, Japan and Prof. Miyagi, Clinical Research Institute, Kanagawa Cancer Center, Japan. They were maintained in RPMI 1640 (Colo201, Colo205, DLD-1, HCT-15, HCT-116, HT-29, KM-12, SW480, and SW620) and DMEM (WiDr) medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. All cells were harvested by centrifugation, rinsed with phosphate buffered saline (PBS), and subjected to total protein extraction in RIPA lysis buffer (50 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% Nonidet P-40, 5 mmol/L dithiothreitol, 10 mmol/L NaF, protease inhibitor cocktail [Sigma]).

Subjects

Colorectal carcinomas (CRCs, n=328) and adjacent non-neoplastic mucosa (NNM, n=329) were collected from surgical resection, and adenoma (n=87) from endoscopic biopsy or polypectomy from the endoscopic biopsy in the Affiliated Hospital, University of Toyama (Japan), and Kouseiren Takaoka Hospital (Japan) between 1993 and 2002. The patients with CRC were 183 men and 145 women (18~90 years, mean=68.6 years). Among them, 130 cases were accompanied by lymph node metastasis. For *in situ* hybridization, colorectal carcinomas (n=107), adenomas (n=87), NNM (n=52), and metastatic foci of lymph node (n=43) and liver (n=26) were collected from the Affiliated Hospital, University of Toyama (Japan) between 2005 and 2009 because there is RNA degradation in old samples. Thirty-two cases of CRC and paired NNM were collected from the First Affiliated Hospital of China Medical University (China) and frozen in -80°C for protein extraction and 27 cases for RNA extraction by homogenization. None of the patients underwent chemotherapy, radiotherapy or adjuvant before surgery. They all provided consent for use of tumor tissue for clinical research and the Ethical Committee approved the research protocol. We followed up the patients by consulting their case documents or by telephone.

Pathology and tissue microarray (TMA)

All tissues were fixed in 10% neutral formalin, embedded in paraffin and cut at 4 μm. These sections were stained by hematoxylin-and-eosin (HE) to confirm their histological characteristics. The staging for each colorectal carcinoma was evaluated according to the Union Internationale Contre le Cancer (UICC) system (Sobin and Wittekind, 2002). Histological architecture of

CRCs was expressed in terms of WHO classification (Hamilton and Aaltonen, 2000). Furthermore, tumor size, depth of invasion, lymphatic and venous invasion were determined.

Representative areas of solid tumors were identified in HE-stained sections of the selected tumor cases and a four mm-in-diameter tissue core per donor block was punched out and transferred to a recipient block with a maximum of 24 cores using a tissue microarrayer (AZUMAYA KIN-1, Japan).

Western blot

Denatured protein was separated on an SDS-polyacrylamide gel (10% acrylamide) and transferred to Hybond membrane (Amersham, Amersham, Germany), which was then blocked overnight in 5% skim milk in TBST (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween 20). For immunobloting, the membrane was incubated for 60 minutes with the rabbit antibody against Bag-3 (Santa Cruz Abcam; 1:1000). Then, it was rinsed by TBST and incubated with anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase (DAKO, Carpinteria, CA93013, USA; 1:1000) for 60 minutes. Bands were visualized with LAS4000 (Fuji, Tokyo, Japan) or X film (Fuji, Japan) by ECL-Plus detection reagents (Santa Cruz, USA). After that, membrane was washed with WB Stripping Solution (pH2-3, Nacalai, Tokyo, Japan) for 1 hour and treated as described above except with anti-ß-actin antibody (Santa Cruz, sc-47778; 1:1000) as an internal control. Densitometric quantification of Bag-3 protein in gastric colorectal

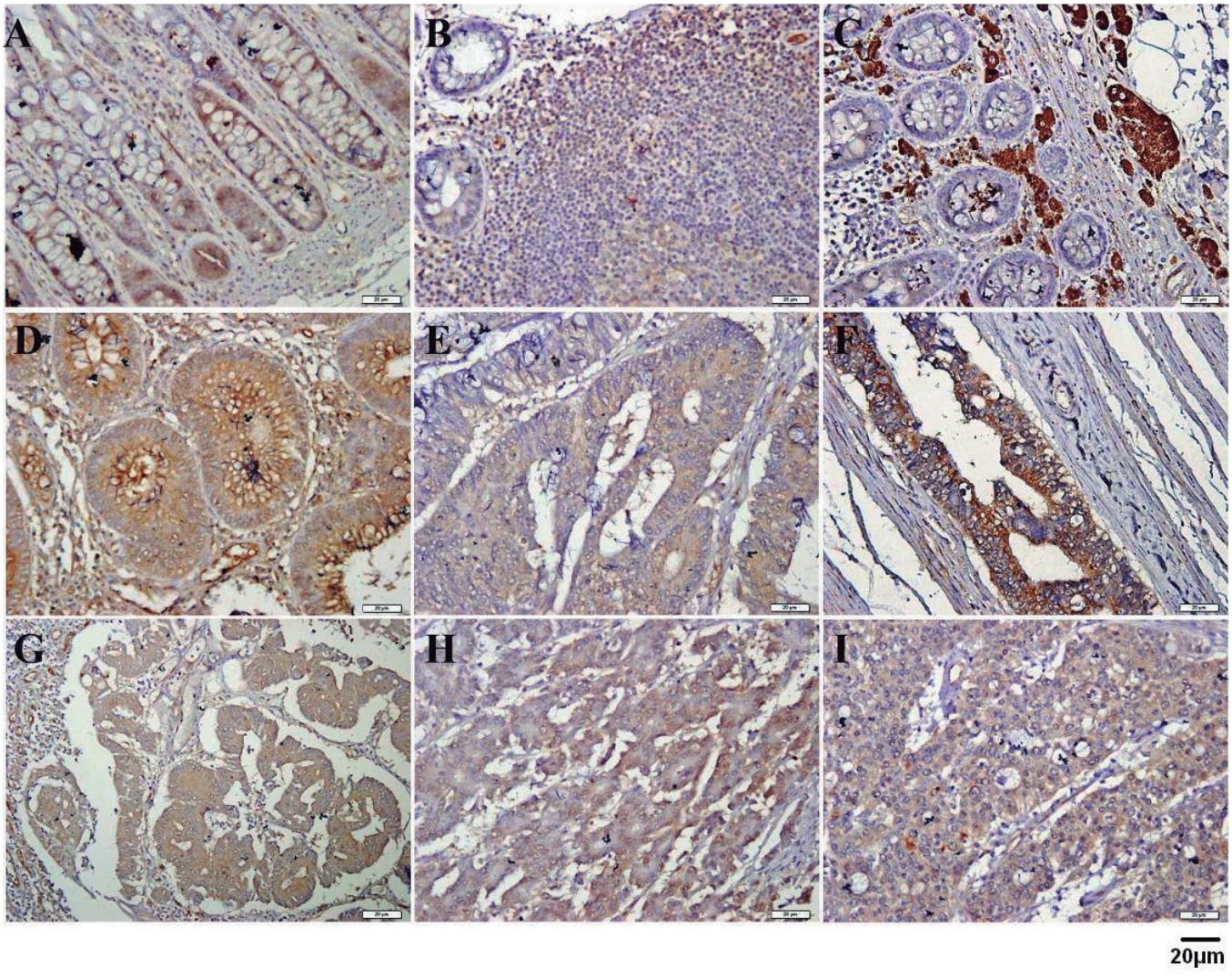

Fig. 1. Bag-3 protein expression in colorectal lesions by immunohistochemistry. Bag-3 was strongly detected in the cytoplasm of colorectal superficial mucosa **(A)**, infiltrating inflammatory cells **(A)**, lymphoid follicle **(B),** macrophages **(C),** adenoma **(D)**, well-differentiated **(E, F)**, moderately- differentiated **(G)** and poorly-differentiated carcinoma **(H, I)**.

samples was performed with ß-actin control using Scion Image software (Scion Corporation, Frederick, MD, USA).

Real-time RT-PCR

Total RNA was extracted from colorectal carcinoma cell lines or tissues using QIAGEN RNeasy mini kit (QIAGEN, Germany) according to the manufacturer's protocol. Two micrograms of total RNA was subjected to cDNA synthesis using the AMV reverse transcriptase and random primer (Takara, Otsu, Japan). Oligonucleotide primers for PCR were designed as 5'-CACCCTTTCCATGTCTATCCC-3' and 5'- CACACTG TTTATCTGGCTGAGT-3' for *Bag-3* (130bp, 631-760, NM_004281.3), and 5'- CAATGACCCCTTCATTG ACC -3' and 5'- TGGAAGATGGTGATGGGATT-3' for GAPDH (135bp, 201-335, NM_ 002046.3). PCR amplification of cDNA was performed in 20 μL mixtures according to the protocol of SYBR Premix Ex Taq™ II kit (Takara).

Immunofluorescence

Cells were grown on glass coverslips, washed twice with PBS, fixed with 4% formaldehyde for 10 minutes at room temperature, and permeabilized with 0.25% Triton X-100 for 10 minutes at room temperature. After washing with PBS, cells were incubated overnight at 4°C with the rabbit antibody against Bag-3 (abnova Abcam; 1:100). They were then washed with PBS and incubated with Alexa Fluor 488 IgG (invitrogen; 1:2000). Nuclei were stained with 1 μg/ml DAPI (Sigma) for 30 minutes at 37°C. Finally, coverslips were mounted with SlowFade® Gold antifade reagent (invitrogen) and observed under a laser confocal scanning microscope (Leica, Germany).

Immunohistochemistry

Consecutive sections were deparaffinised with xylene, rehydrated with alcohol, and subjected to antigen retrieval by irradiating in target retrieval solution (TRS, DAKO, USA) for 15 minutes with a microwave oven (Oriental rotor Lmt. Co., Tokyo, Japan). The sections were quenched with 3% hydrogen peroxide in absolute methanol for 20 minutes to block endogenous peroxidase activity. Five percent bovine serum albumin was then applied for 5 minutes to prevent non-specific binding. The sections were incubated with antibodies against Bag-3 (Abnova Abcam; 1:200) and GRP94 (Santa Cruz; 1:100) for 15 minutes, then treated with the anti-rabbit or anti-goat conjugated to horseradish peroxidase (DAKO, USA) antibodies for 15 minutes. All the incubations were performed in a microwave oven to allow intermittent irradiation as described previously (Kumada et al., 2004). After each treatment, the slides were washed with TBST three times for 1 minute.

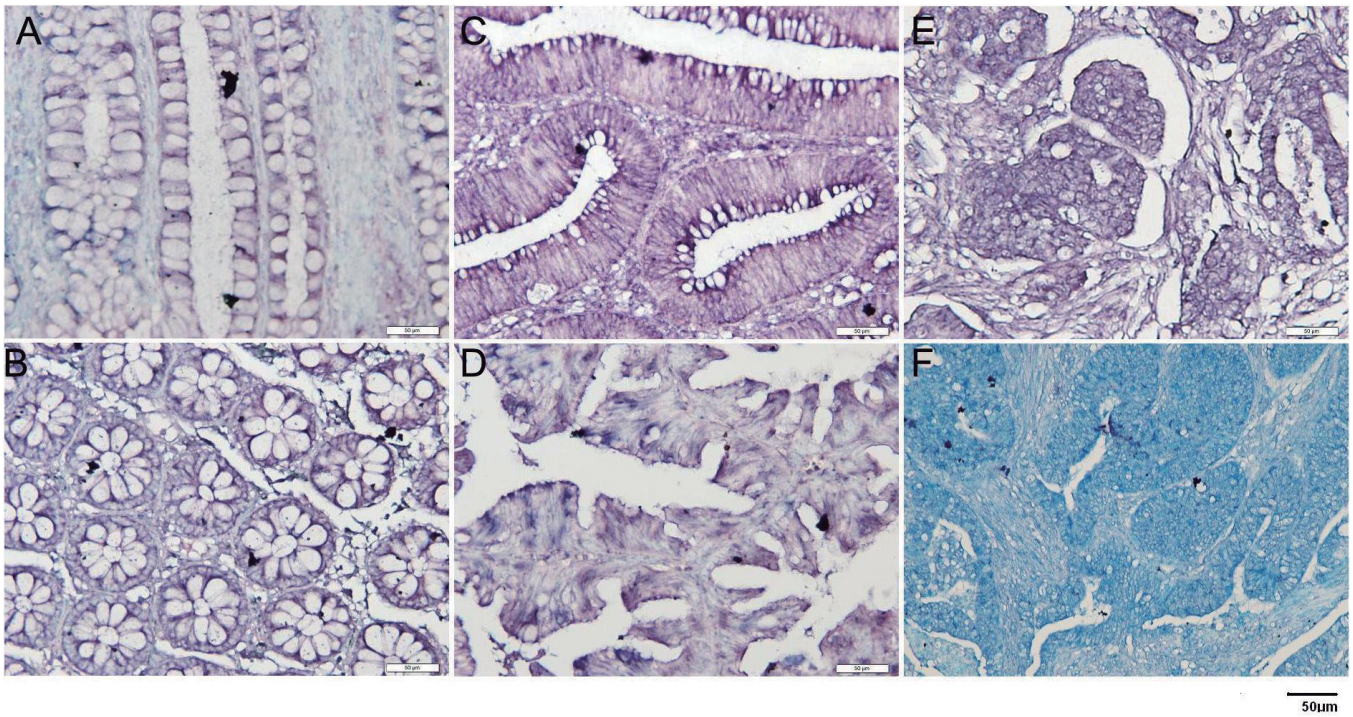

Fig. 2. *Bag-3* mRNA expression in colorectal carcinogenesis by *in situ* hybridazation. *Bag-3* mRNA was positively observed in the cytoplasm of colorectal gland **(A, B),** tubular **(C)** and villous **(D)** adenomas, and adenocarcinomas **(E).** There was no positive signal of *Bag-3* mRNA in negative control of colorectal adenocarcinoma **(F)**.

Binding sites were visualized with 3, 3'-diaminobenzidine. After being counterstained with Mayer's hematoxylin, the sections were dehydrated, cleared and mounted. Omission of the primary antibody was used as a negative control.

As indicated in Fig. 1, Bag-3 or GRP94 (Takahashi et al., 2011) protein was positively localized in the cytoplasm. One hundred cells were randomly selected and counted from 5 representative fields of each section blindly by two independent observers (Yang X and Zheng HC). Inconsistent data were confirmed by both persons until final agreements were reached. The positive percentage of counted cells was graded semi-quantitatively according to a four-tier scoring system: negative (-), 0~5%; weakly positive (+), 6~25%; moderately positive (++), 26~50%; and strongly positive (+++).

In situ hybridization

To perform RNA-DNA *in situ* hybridization (ISH) for *Bag-3*, a digoxygenin-labeled *Bag-3* probe was made in 35-cycle PCR using *Bag-3* primers(5'- CACCCTTT CCATGTCTATCCC-3' and 5'- CACACTGTTTATCTG GCTGAGT -3', 130bp, 631-760, NM_004281.3) and 15ng template DNA of 30-cycle products from the DLD-1 cDNA using Pfu polymerase (Stratagene, USA). Four-μm-thick sections were deparaffinized and digested with 20 μg/mL proteinase K in 50 mmol/L Tris-HCl at 37°C for 10 minutes. Then 20 μL of a 1: 20 probe dilution in hybridization buffer (22mmol/L Tris-HCl, pH7.4, 2.75 mmol/L ethylenediaminetetraacetic acid, 660 mmol/L

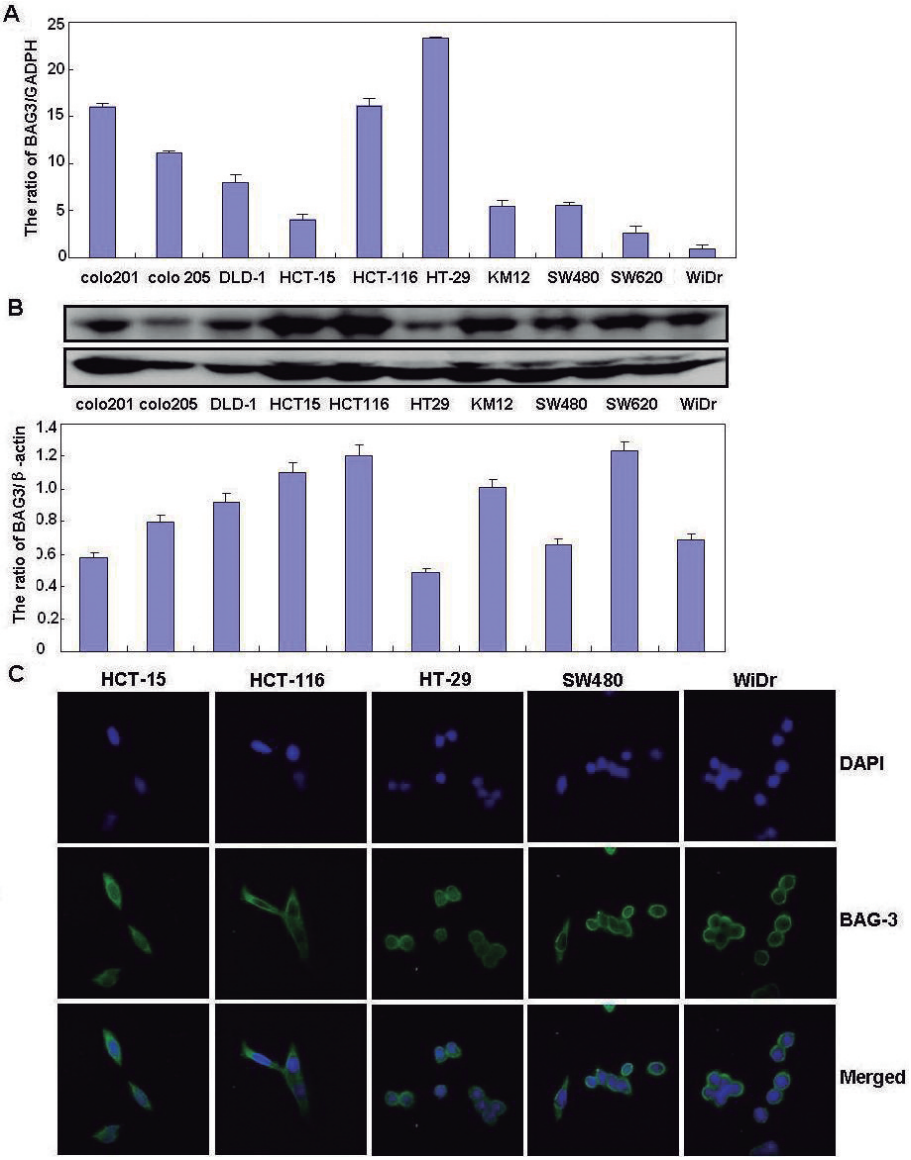

Fig. 3. Bag-3 expression in colorectal carcinoma cell lines. **A.** Bag-3 mRNA was detected and in colorectal carcinoma cell lines with an internal control of GAPDH by real-time RT-PCR. The ratio of Bag-3/GAPDH is considered as "1" in WiDr cells. Lane #1, Colo201; Lane #2, Colo205; Lane #3, DLD-1; Lane #4, HCT-15; Lane #5, HCT- 116 ;Lane #6, HT-29; Lane #7, KM-12; Lane #8, SW480; Lane #9, SW620; Lane #10, WiDr. **B.** The cytosolic protein was loaded and probed by the anti-Bag-3 antibody (61kDa) with ß-actin (42 kDa) as an internal control. Densitometric quantification was performed to evaluate Bag-3 protein expression level. Lane #1, Colo201; Lane #2, Colo205; Lane #3, DLD-1; Lane #4, HCT-15; Lane #5, HCT-116; Lane #6, HT-29; Lane #7, KM-12; Lane #8, SW480; Lane #9, SW620; Lane #10, WiDr. **C.** HCT-15, HCT-116, HT-29, Sw480 and WiDr carcinoma cells were subjected to immunofluorescence staining of Bag-3 protein, which is distributed to the cytoplasm (Green, Bag-3; blue, DAPI).

NaCl, 1x Denhardt solution, 5.5% dextran sulfate, 0.33% dimethyl sulfoxide, 0.55% ethoquad18/25 and 44% deionized formamide) was added to each slide. After coverslipping, heating at 95°C for 5 minutes, and incubation overnight in a humidified chamber at 37°C, sections were rinsed for 10 minutes in TBST and incubated with anti-digoxygenin antibody conjugated with alkaline phosphatase (AP, Roche Diagnostics, Germany) for 20 minutes at 37°C. The slides were then washed for 5 minutes and immersed in solution II (100 mmol/L Tris-HCl pH9.5, 100 mmol/L NaCl and 50 mmol/L $MgCl_2$) for 30 minutes, followed by exposure to NBT(nitro-blue tetrazolium chloride)/ BCIP (5-Bromo-4- Chloro-3'-Indolylphosphatase p-Toluidine salt) as a chromogen. Finally, counterstaining was performed using methyl green for 2 minutes.

As indicated in Fig. 2, *Bag-3* mRNA signal was positively localized in the cytoplasm. One hundred cells were randomly selected and counted from 5 representative fields of each section blindly by two independent observers (Yang X and Zheng HC). Inconsistent data were confirmed by both persons until final agreements were reached. The positive percentage of counted cells that was more than 5% was considered as positive (+).

Statistical analysis

Statistical evaluation was performed using *Spearman's* correlation test to analyze the rank data, chi-square test to compare the different rate, and Wilcoxon test to differentiate the means of different groups. Kaplan-Meier survival plots were generated and comparisons between survival curves were made with the log-rank statistics. *Cox's* proportional hazards model was employed for multivariate analysis. SPSS 10.0 software was applied to analyze all data and $P<0.05$ was considered statistically significant.

Results

Bag-3 expression in colorectal carcinoma cell lines or tissue samples

To check *Bag-3* mRNA expression, we employed real-time RT-PCR and observed lower expression of Bag-3 in HCT-15, KM12, SW480, SW620 and WiDr cells (Fig. 3A). As shown in Fig. 3B, Bag-3 protein was comparatively lower in HT-29, SW480 and WiDr cells. The immunofluorescence staining showed that Bag-3 was distributed in the cytoplasm of HCT-15, HCT-116,

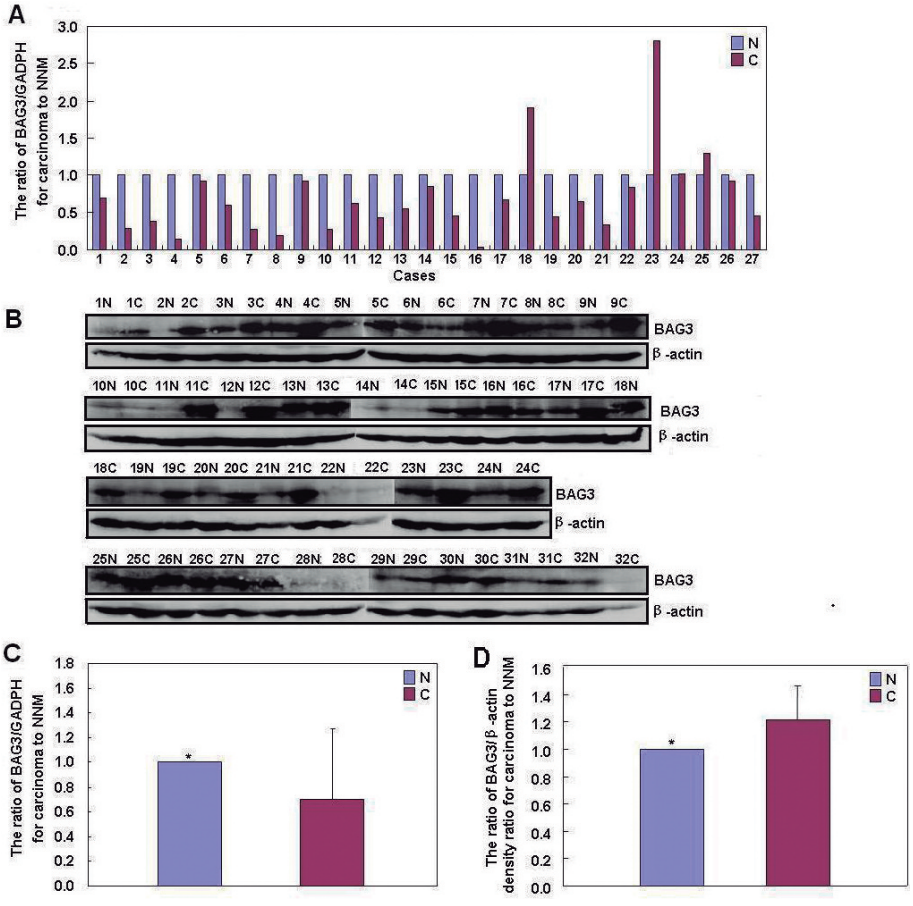

Fig. 4. Bag-3 expression in colorectal carcinoma and matched non-neoplastic mucosa. **A.** Bag-3 mRNA was amplified in colorectal carcinoma and paired non-neoplastic mucosa (NNM) with an internal control of GAPDH by real-time PCR. **B.** Cell lysate was loaded and probed with anti-Bag-3 (Panel 1, 61kDa) with ß-actin (Panel 2, 42 kDa) as an internal control. N, non-neoplastic mucosa; C, carcinoma. **C.** There was more Bag-3 mRNA expression in colorectal NNM than corresponding carcinoma (*, $P<0.05$). **D.** Bag-3 protein was highly expressed in colorectal carcinoma in comparison with paired NNM (*, $P<0.05$).

HT-29, SW480 and WiDr cell lines (Fig. 3C). Among 27 cases of frozen colorectal samples, *Bag-3* mRNA was less expressed in carcinoma than adjacent NNM by quantitative RT-PCR (P<0.05, Fig. 4A,C), while Bag-3 showed the opposite trend by Western blot (P<0.05, Fig. 4B,D).

As shown in Fig. 1, Bag-3 was strongly detected in the cytoplasm of the superficial mucosa, infiltrating inflammatory cells, lymphoid follicle, macrophages, adenoma, well-, moderately- and poorly-differentiated carcinoma. Statistically, Bag-3 expression was detectable in colorectal NNM (16.1%, 53/329), adenoma (31.0%, 27/87), and carcinoma (54.3%, 178/328), respectively. Statistically, Bag-3 expression was seen to gradually decrease in samples from carcinoma, adenoma to NNM (P=0.001, Table 1).

To confirm *Bag-3* mRNA expression, ISH was also employed on TMA of the colorectal carcinoma and adjacent mucosa (Fig. 2). *Bag-3* mRNA was detected in colorectal NNM (64.6%, 34/52), adenoma (70.0%, 61/87), carcinoma (42.1%, 43/107), metastatic carcinoma in lymph node (83.7%, 36/43) and liver (88.5%, 23/26). Statistically, its positive rate was higher in colorectal NNM than carcinoma (P<0.05). Metastatic carcinoma more frequently expressed *Bag-3* mRNA in lymph node and liver than in primary carcinoma (P<0.001).

Table 1. Bag-3 expression in colorectal carcinogenesis.

Groups		Bag-3 expression				
	n	-	+	++	+++	PR(%)
Non-neoplastic mucosa	329	276	47	6	0	16.1
Adenoma	87	60	10	10	7	31.0
Carcinoma	328	150	59	63	56	54.3*

* P=0.001.PR, positive rate

Table 2. Relationship between Bag-3 expression and clinicopathological features of colorectal carcinomas.

Clinicopathological features		Bag-3 expression					
	n	-	+	++	+++	PR(%)	*P* value
Age(years)							0.550
<65	126	59	25	21	21	53.2	
≥65	200	89	34	42	35	55.5	
Sex							0.146
Male	183	91	31	31	30	50.3	
Female	145	59	28	32	26	59.3	
Tumor size(cm)							0.218
<5	218	94	42	41	41	56.9	
≥5	109	55	17	22	15	49.5	
Differentiation degree							0.090
Well-differentiated	165	85	25	28	27	48.5	
Moderately-differentiated	149	60	32	29	28	59.7	
Poorly-differentiated	14	5	2	6	1	64.3	
Depth of invasion							0.354
T_{is-1}	27	13	8	3	3	51.9	
T_{2-4}	297	134	51	59	53	54.9	
Lymphatic invasion							0.396
-	227	102	37	45	43	55.1	
+	92	42	22	16	12	54.3	
Venous invasion							0.161
-	271	118	51	53	49	56.5	
+	48	26	8	8	6	45.8	
Lymph node metastasis							0.786
-	193	90	34	35	34	53.4	
+	130	57	25	27	21	56.2	
TNM Staging							0.015
0-I	29	17	8	3	1	41.4	
II-IV	243	103	41	56	43	57.6	
GRP94 expression							0.001
-~+	214	111	34	38	31	48.1	
++~+++	88	27	19	20	22	69.3	

CRC, colorectal carcinoma; PR, positive rate; T_{is}, carcinoma *in situ*; T_1, lamina propria and submucosa; T_2, muscularis propria; T_3, subserosa and exposure to serosa; T_4, invade other organs or perforate visceral peritoneum; UICC, Union Internationale Contre le Cancer.

The relationship between Bag-3 expression and clinicopathological and prognostic parameters of colorectal carcinoma

As summarized in Table 2, there was a positive correlation between Bag-3 expression and TNM staging or GRP94 expression (P<0.05), but no relationship to patient age or sex, tumor size, depth of invasion, lymphatic or venous invasion, lymph node metastasis or differentiation of CRCs (P>0.05).

Follow-up information was available on 280 colorectal carcinoma patients for periods ranging from 0.2 months to 12.1 years (median=66.5 months). Survival curves for patients with CRCs were stratified by Bag-3 expression (Fig. 5). Univariate analysis using *Kaplan-Meier* method indicated that Bag-3 expression was not related to the cumulative survival rate of patients with overall, early or advanced CRC (P>0.05). Multivariate analysis using Cox's proportional hazard model indicated that lymphatic invasion, venous invasion, and lymph node metastasis (P<0.05), but not age, sex, tumor size, depth of invasion, TNM staging, differentiation degree or Bag-3 expression, were independent prognostic factors for overall CRCs (P>0.05, Table 3).

Discussion

Increasing evidence suggests that the biochemical events of apoptosis are controlled through the expression of many cellular proteins, including inducers (Bax, Bcl-Xs, Bad, Bak, and Bik) and inhibitors (Bcl-2, Bag-1, Bag-3, Bcl-XL, and Mcl-1) of cell death (Zheng et al., 2010). Bag-3 is reported to play an important role in protein homeostasis, cell survival, actin organization, cell adhesion, cell motility and tumor metastasis (Kassis et al., 2009; McCollum et al., 2009). The current study demonstrated that Bag-3 was distributed in the cytoplasm of colorectal epithelial or carcinoma cells using Bag-3 antibody from Abnova cam, but its nuclear localization was also observed using the antibody from

Table 3. Multivariate analysis of clinicopathological variables for survival with colorectal carcinomas.

Clinicopathological parameters	Relative risk (95%CI)	P value
Age (≥65years)	1.395 (0. 882-2.207)	0.154
Sex (female)	1.207 (0.688-1.739)	0.704
Tumour size (≥5cm)	0.996 (0.636-1.561)	0.987
Diff (well-mod/poor)	1.070 (0.683-1.676)	0.769
Depth of invasion (T2-4)	2.424 (0.287-20.486)	0.416
Lymphatic invasion (+ ~)	1.731 (1.077-2.782)	0.024
Venous invasion (+ ~)	1.965 (1.124-3.436)	0.018
Lymph node metastasis (+ ~)	3.106 (1.949-4.948)	<0.001
TNM staging (II-IV)	1.619 (0.456-5.752)	0.457
Bag-3 expression (+ ~ +++)	0.834 (0.528-1.316)	0.436

CI, confidence interval; TNM, tumor-node-metastasis.

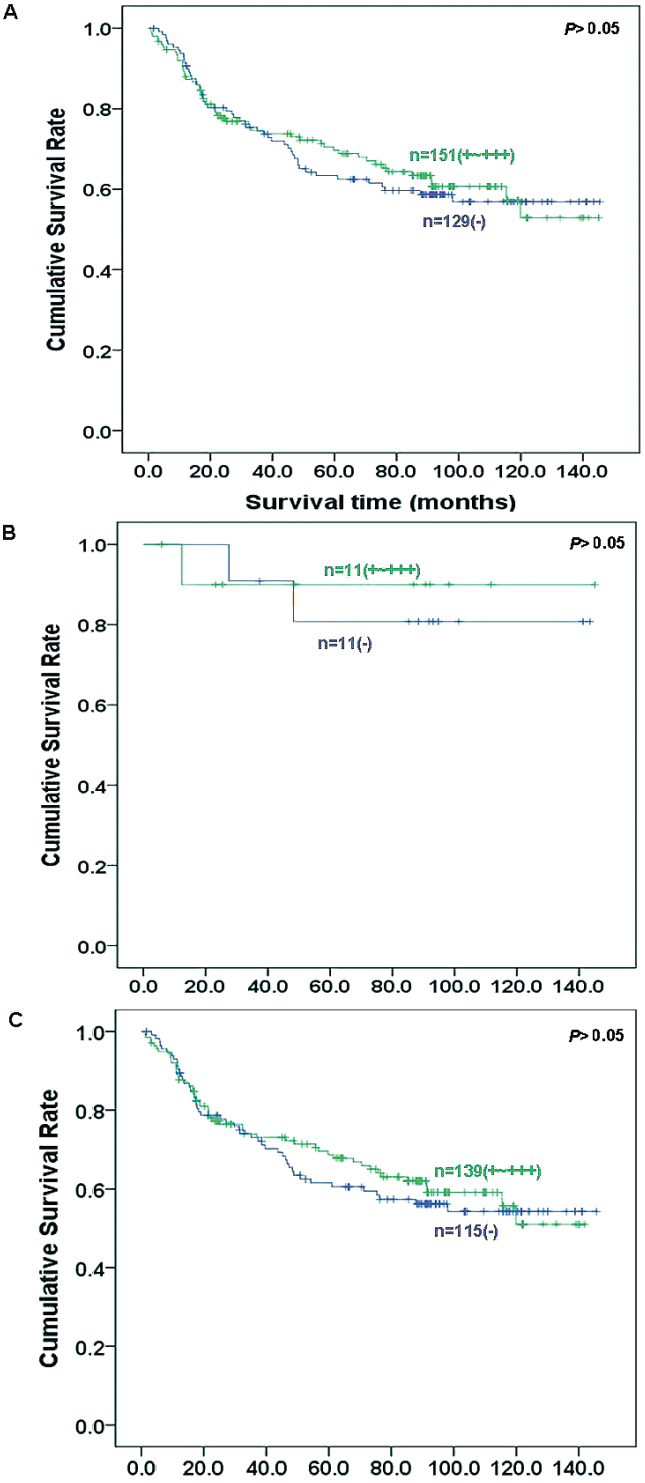

Fig. 5. Correlation between Bag-3 expression and prognosis of the colorectal carcinoma patients. Kaplan-Meier curves for cumulative survival rate of patients with overall **(A)**, early **(B)** or advanced **(C)** colorectal carcinomas according to Bag-3 expression.

Santa Cruz (data not shown). Immunohistochemically, Bag-3 protein was strongly detected in the cytoplasm of the superficial mucosa, infiltrating inflammatory cells, lymphoid follicle, macrophages, adenoma and adenocarcinoma, indicating that Bag-3 expression has cell-specific property, whose biological effects should be further investigated in these cells.

Here, Bag-3 expression was seen to gradually decrease in samples from carcinoma, adenoma to NNM according to immunostaining findings. Western blot also showed that Bag-3 was more expressed in carcinoma than matched NNM. Combined with both results, we could conclude that up-regulated Bag-3 expression might contribute to colorectal carcinogenesis. Reportedly, Bag-3 was highly expressed in human malignancies like thyroid carcinoma or glioblastomas in comparison with its normal counterpart, in line with our observation (Chiappetta et al., 2007; Festa et al., 2011). Jacobs and Marnett (2009) found silencing Bag-3 expression caused a dramatic reduction in Bcl-X(L), Mcl-1, and Bcl-2 protein levels in colon cancer cells and increased apoptosis via specific interactions between Bag-3, Hsp70, and the Bcl-2 family member, Bcl-X(L), suggesting a unique role of Bag-3 in facilitating cancer cell survival during pro-apoptotic stress by stabilizing the level of Bcl-2 family proteins. To check transcriptional regulation of Bag-3, RT-PCR and ISH were employed and they demonstrated lower *Bag-3* mRNA in colorectal carcinoma, compared with paired NNM, opposite to the findings at protein level. The paradoxical phenomemon could be explained by the difference in mRNA stability, translation and protein degradation of Bag-3 between colorectal carcinoma and NNM. In contrast, Liao et al. (2001) documented that *Bag-3* mRNA was expressed at moderate to high levels in pancreatic cancer samples, but at low levels in normal pancreas tissues. The inconsistent results demonstrated that aberrant *Bag-3* mRNA expression might be of tissue specificity in cancer tissues. To my knowledge, a large amount of enzyme in pancreas leads to rapid RNA degradation in pancreas (Vilardell et al, 2010) while RNA is comparatively stable in pancreatic cancer tissue, which might be hypothesized to cause Bag-3 overexpression in pancreatic cancer. Different from our finding, there is another report about no difference in *Bag-3* mRNA expression between colonic carcinoma and matched mucosa by Northern blot (Liao et al, 2001), which might be due to distinct methodologies because of our mRNA detection by real-time PCR.

To clarify the clinicopathological significance of Bag-3 expression, IHC were performed on TMA. It was found that Bag-3 expression was positively correlated with TNM staging. *Bag-3* mRNA was more frequently expressed in metastatic carcinoma in liver and lymph node, but we could not explain why *Bag-3* mRNA, which was down-regulated in CRCs, positively correlated with their aggressiveness. However, Staibano et al. (2010) found that cytoplasmic Bag-3 expression showed a progressive decrease of the expression level from well- to low-differentiated carcinoma, coupled with the loss of polarization of the signal in metastasizing cases of prostate carcinomas. Suzuki et al. (2011) reported that Bag-3 positivity was significantly higher at advanced clinical stages of ovarian cancer than at early stages. Further investigation indicated that Bag-3 interacted with MMP-2 in cultured ovarian cancer cells, and that knockdown of Bag-3 expression down-regulated MMP-2 expression and diminished cell motility and invasiveness. In combination with these findings, we speculate that the role of Bag-3 in invasion and metastasis of malignancies might be of tissue specificity.

GRP94 is representative of the HSP90 family of stress-induced proteins and plays an important role in stabilizing calcium homeostasis and suppressing cell death. The induction of GRP94 expression may be a defense mechanism for the survival of cancer cells exposed to stress, glucose deprivation, hypoxia or lack of nutrients (Zheng et al., 2008). Jung et al. (2010) found that Bag-3 knock-down resulted in an increase in the cell death rate of C6 cells after oxygen-glucose deprivation, accompanied by accumulation of reactive oxygen species. Combined with these findings, we believe that GRP94 and *Bag-3* might be involved in the cell survival in response to microenvironmental oxygen and nutrient deprivation, which could explain the positive relationship between GRP78 and Bag-3 expression in CRCs.

Until now, there was yet no paper describing the prognostic significance of Bag-3 expression in malignancies. In the present study, the relevance of Bag-3 expression for prognosis is discussed, but Bag-3 expression is not linked to the prognosis of patients with CRC that were stratified by the depth of invasion. Multivariate analysis demonstrated that lymphatic invasion, venous invasion, and lymph node metastasis, but not age, sex, depth of invasion, tumor size, venous invasion, TNM staging, differentiation, or Bag-3 expression, were independent prognostic factors for carcinomas. These findings suggested that Bag-3 expression is not an indicator for the prognosis of colorectal carcinoma patients.

In summary, our study indicated that aberrant *Bag-3* expression might impact the malignant transformation of colorectal epithelial cells and should be considered as a good biomarker for colorectal carcinogenesis and subsequent progression. Nevertheless, the biological functions of Bag-3 protein in CRCs need further investigation.

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