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Original Article

eIF3C as a prognostic marker of colorectal cancer

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Abstract: Eukaryotic translation initiation factor 3C (eIF3C) is a component of the eukaryotic translation initiation factor 3 (eIF3) complexes, which has a crucial role in the translation initiation process. As we know, translational control plays a key role in the complex mechanism of cancer development and progression. Besides, eIF3C down-regulation decreases the global protein synthesis and causes a polysome run-off. In vitro and clinical trials were made to prove the relationship of eIF3C and colorectal cancer (CRC). We analyzed the eIF3C expression in a tissue microarray (TMA), which contained 198 colorectal cancer tissues and paired adjacent normal mucosa. The immunohistochemistry was used to assess the relative expression level of eIF3C and its value as a prognostic marker. SPSS was used to analysis the data to figure out the association between eIF3C and CRC. eIF3C was found highly expressed in CRC cells by in vitro experiments and clinical researches, and it can influence tumor proliferation, colony formation and cell cycle. In addition, eIF3C is an independent risk factor for CRC patients both OR and RFS. All in all, high eIF3C expression may contribute to tumor progression and predict poor clinical staging of colorectal cancer.

Keywords: Colorectal cancer, eIF3C, prognosis

Introduction

Colorectal cancer (CRC) is a normal gastrointestinal tumor, it represents significant morbidity and mortality although the incidence of CRC has been declining over the past 20 years in the United States [1]. The morbidity of CRC ranks the third and the mortality ranks the fourth in the world. In some Asian counties, like China, the CRC morbidity keeps increasing [2].

With the fast development of surgery, chemotherapy and targeted therapy, the prognosis of CRC has been dramatically improved. However, the 5-year-survival rate of advanced CRC is still less than 50%. The metastasis has been believed as one of the main reasons of death. About 10% to 30% patients with radical surgeries will get liver metastasis eventually [3]. The TNM staging of CRC is a critical indication for the prognosis and to make treatment schedules, and thus is useful for most of the patients. However, we noticed that each patient has his/her own pathological and physiological specialties; even the patients at the same TNM staging could have a significant difference in overall survivals and recurrence-free survivals. Because of this kind of heterogeneity, it is necessary to search representative markers that closely related to the tumor biological behavior and clinical prognosis to make a precise and personalized treatment schedule for each patient. Translation level and transcription level are widely accepted to play an imperative role in gene expression, and eIF3C might be an important part in translation and transcription [4]. Although the expression level of eIF3C is low, it exists in most of the cells. And the changes of eIF3C could be special for CRC, because of the complicated 5'UTR.

In this work, we investigate the influence of eIF3C on the cells and on the prognosis of the CRC patients through in vitro experiments, produced the TMAs, immunohistochemistry, and analysis the follow up data with multivariate Cox regression.

Materials and methods

Patients and specimens

Tissue specimens were collected from colorectal cancer patients who underwent radical

operation between May 2011 and July 2013 at Fudan University Huashan Hospital. None of these patients received adjuvant therapy before the surgery. The chemotherapy after surgery is similar. 202 formalin-fixed, paraffin-embedded tissues of CRC and normal colorectal were applied for histological and immunohistochemical analysis. All the basic information of the 202 patients was collected, such as age, gender, BMI, ASA levels, cancer location, pathology, blood markers, surgery procedures, patients' survival and tumor recurrence. Two pathologists who were blinded to clinical information, observed and analyzed the specimens to make sure of the histological stage and pathological type. Tumor staging was distinguished by the American Joint Committee on Cancer (AJCC) classification system based on the tumor size (T), lymph node involvement (N), and distant metastasis (M) [5]. The disease-free survival (DFS) was defined as the interval from the surgery to clinically or radiologically proven recurrence or metastasis whereas the overall survival (OS) was defined as the interval from initial surgery to death. The follow-up was carried out according to the guidelines of Office for Human Research Protections (OHRP) Human Subject Protections and the end date was October 29, 2016, and 10 patients were lost to follow up. The guidelines of Office for Human Research Protections (OHRP) Human Subject Protections

Tissue microarray (TMA) construction

TMA was constructed with the Zhuoli Biotechnology Co. Ltd. (Shanghai, China). After histologic examination of H&E-stained sections of formalin-fixed, paraffin-embedded tumor samples was checked by an experienced pathologist, the core positions of CRC and normal colorectal tissue were determined. Then, we obtained the samples with the help of a 1.5 mm diameter punch instrument (BEECHER TISSUE ARRAYER, America), and 2 cores were collected for each sample to make sure there was no vital difference caused by heterogeneity. Finally, we arranged these cores in the paraffin block.

Immunohistochemistry staining

The tissues of interest collection were formalin-fixed and paraffin-embedded. Briefly, immunostaining was performed using a Booster Tech Company kit (Shanghai, China). After antigen retrieval, sections were incubated overnight at 4°C with the primary antibody against eIF3C (Anti-eIF3C antibody ab36766, Abcam; Cambridge, MA, USA; diluted 1:100). The slides were then incubated with secondary antibody for 30 min at room temperature. The results of final immunohistochemistry score for each sample was calculated by multiplying the intensity score and the percentage of positive cells. Two pathologists blinded to the patient clinical information read the results separately. The degree of staining was classified into four groups according to the staining intensity and the percentage of positive tumor cells: negative, weakly positive, positive and strongly positive, representing negative to the highest eIF3C expression, respectively [6]. The negative and weakly positive groups were concluded as low expression group; the positive and strongly positive groups were concluded as high expression group.

Cell culture

Six CRC cell lines, RKO, HCT116, SW480, SW620, LoVo and HEK293 were purchased from American Type Culture Collection (ATCC). Cells were routinely cultured in complete Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator containing 5% CO_2. Cells count was performed using a Cellomics Array Scan® VTI HCS reader.

Lentiviral-mediated RNA interference (RNAi)

The sequences of the siRNA for eIF3C and control siRNA were synthesized as 5'-GAC CAT CCG TAA TGC CAT GAA-3' and 5'-TTC TCC GAA CGT GTC ACG T-3', respectively. These nucleotide sequences were inserted into the plasmid using the shRNA expressing vector pFUGW (Hollybio, Shanghai, China) and lentiviral packing vectors pVSVG-1 and pCMVΔR8.92 (Hollybio, Shanghai, China). The identities of the generated lentiviral based shRNA expressing vectors were confirmed by DNA sequencing. For the transfection, HEK293T cells (1×10^7) were seeded in 10 cm dishes and cultured for 24 h to reach 70%-

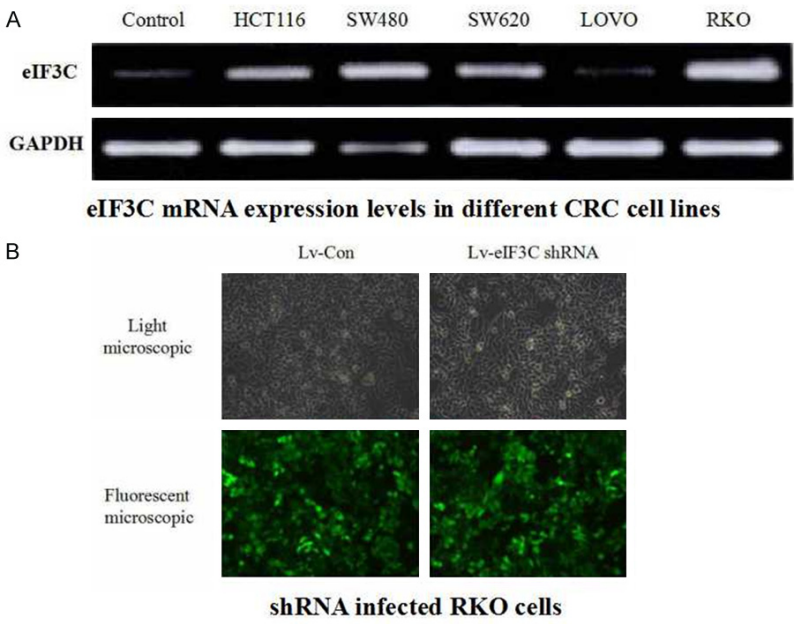

Figure 1. eIF3C mRNA expression levels in different CRC cell lines and successful infection of RKO cells. A. eIF3C expression levels of mRNA in different CRC cell lines; B. Light microscopic (top) and fluorescent microscopic (bottom) pictures of RKO cells.

80% confluence. Two hours before transfection, the medium was replaced with serum-free DMEM and 3 plasmids, including 20 μg of silencing sequence or control sequence, 15 μg of packaging vector pCMVΔR8.92 and 10 μg of VSVG-1 plasmid, were added to 200 μl of opti-MEM and 15 μl of Lipofectamine 2000. The mixture was added to the cells and incubated for 8 hours prior to replacement with 10 ml of DMEM medium containing 10% FBS. The supernatant was collected after 48 h of transfection; then the lentiviral particles were harvested by collecting the precipitate of this supernatant after ultra-centrifugation (4000×g) at 4°C for 10 min.

On the infection of RKO cells with eIF3C shRNA or control shRNA, cells were seeded into 96-well plates (50000 cells/well). After 24 h of incubation, the culture medium was replaced with opti-MEM medium containing lentivirus. After a further 24 h, the virus-containing incubation medium was replaced with fresh medium and incubated for 72 h. The success of infection was examined by counting the green fluorescence emitted by the green florescence protein (GFP) within the lentivirus particles under fluorescence microscopy.

Reverse transcription polymerase chain reaction (RT-PCR) and Western-blotting (WB)

TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for total RNA extraction. cDNA synthesis was performed using MMLV cDNA kit (Invitrogen; Thermo Fisher Scientific, Inc) and using this as the PCR template. All these procedures are in accordance with the manufacturer's agreement. The following primers (Sangon Biotech, Shanghai, Co., Ltd.) were used: eIF3C, sense primer 5'-AGA TGA GGA TGA GGA TGA GGA C-3', antisense primer 5'-GGA ATC GAA GAT GTG GAA CC-3'; and GAPDH, sense 5'-TGA CTT CAA CAG CGA CAC CCA-3', and antisense 5'-CAC CCT GTT GCT GTA GCC AAA-3'. GAPDH was measured as an internal control for mRNA. All samples were normalized to internal controls and fold changes were calculated based on relative quantification using the 2-ΔΔ Cq method.

We used Western blotting to evaluate the expression of eIF3C protein levels in post-infected CRC cells. The protein was isolated after the cells were collected, and we used the Lowry method to measure the protein content. The protein concentration of each sample was adjusted to 2 μg/μl. Then, 20 μl of collected protein was loaded onto a 12 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrophoresed at 60 V for 4 h. The gel was transferred to polyvinylidene difluoride (PVDF) membrane. The next step was detecting the proteins by respective antibodies using electrochemiluminescence (ECL) kit, and then exposing to X-ray. The bands on X-ray films were quantified with an Image Quant densitometric scanner. GAPDH was used as control.

Cell proliferation

Cells were trypsinized at the logarithmic phase and resuspended in complete medium. After

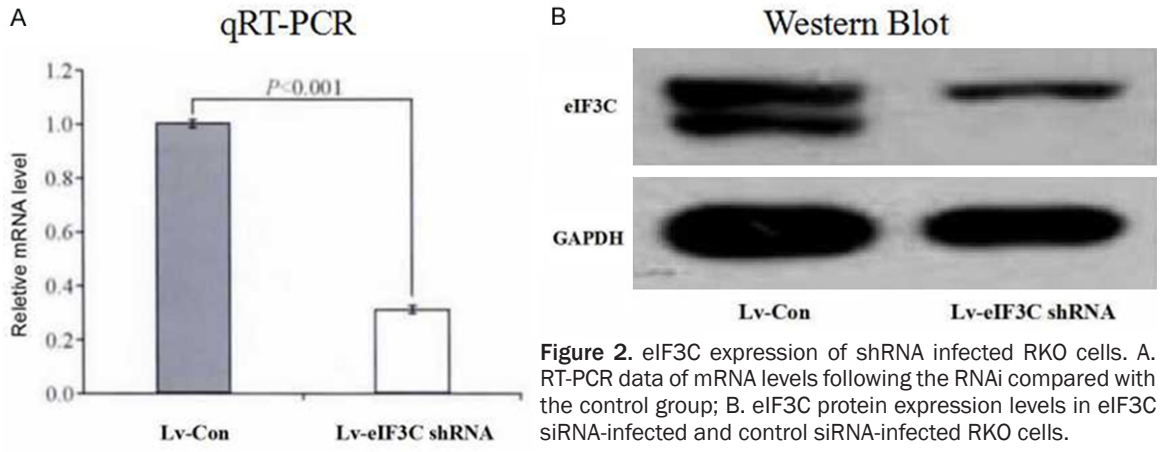

Figure 2. eIF3C expression of shRNA infected RKO cells. A. RT-PCR data of mRNA levels following the RNAi compared with the control group; B. eIF3C protein expression levels in eIF3C siRNA-infected and control siRNA-infected RKO cells.

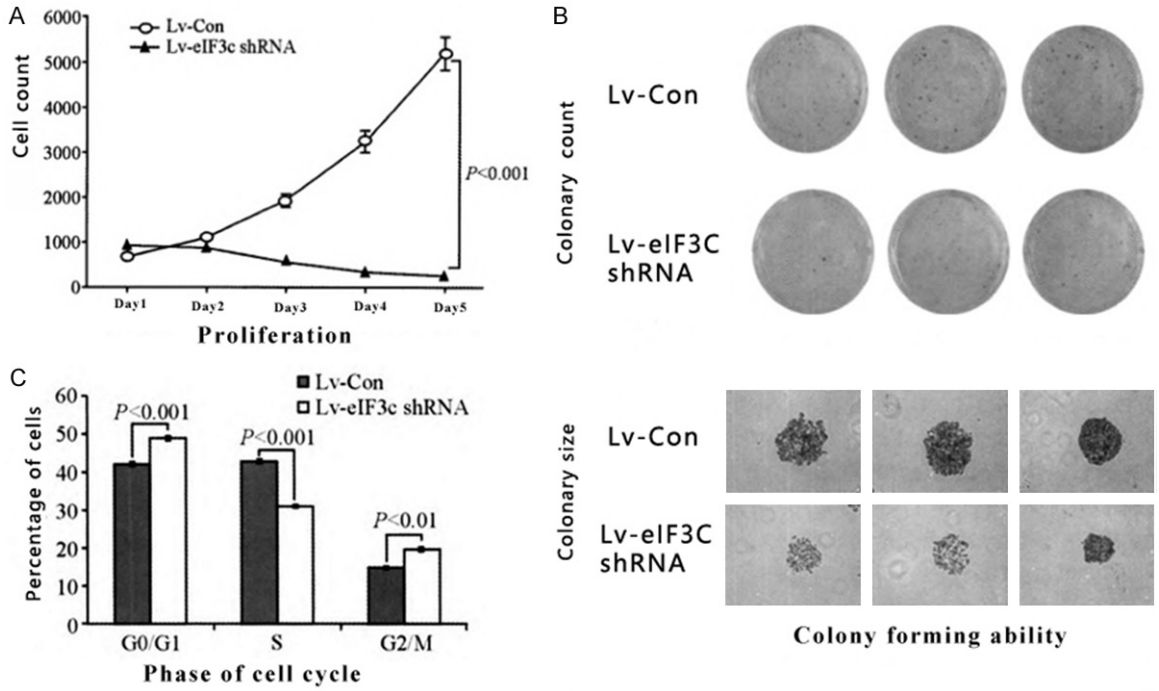

Figure 3. The proliferation, colony formation and cell cycle of shRNA infected RKO cells. A. The number of cells every day; B. The colony numbers at the fifth day; C. Percentages of RKO cells at different phases of the cell cycle at 24 h.

cells were seeded into the 96 well plates (500 cells/well), the proliferation level of RKO cells after eIF3C siRNA infection was analyzed everyday by the Cellomics machine, which counting the number of viable cells by their green fluorescence emission. Each examination repeats 3 times.

Plate colony formation assay

Five days after infection, we collected the RKO cells and seeded them into the 6 well plates (500 cells/well), besides we changes the culture medium every 3 days. After 2 weeks incubation, we fixed the colonies with 4% paraformaldehyde solution. In the subsequent step, we stained the cells with Giemsa for 20 min. The plates were photographed, and the colonies (> 50 cells) were counted by fluorescence microscope.

Statistical analysis

All data are expressed as mean ± standard deviation (SD) of three independent experiments and the error bars represent the SD. In

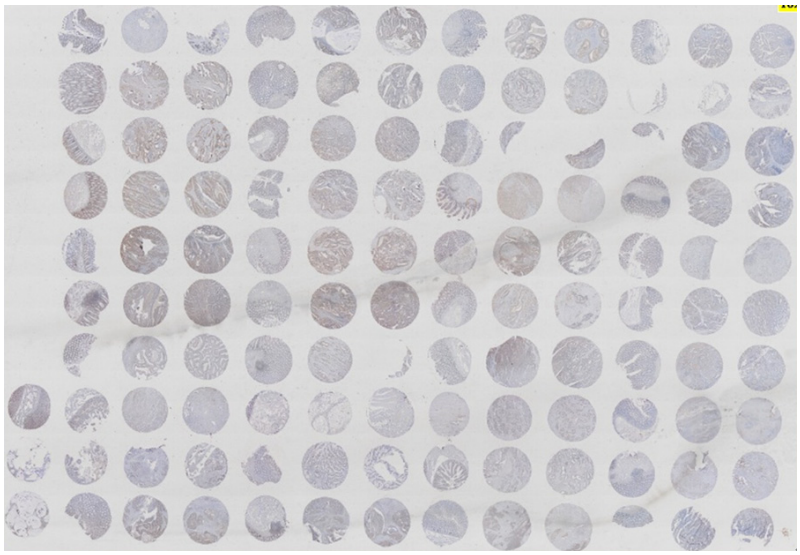

Figure 4. The TMA of CRC tissues and adjacent normal mucosa.

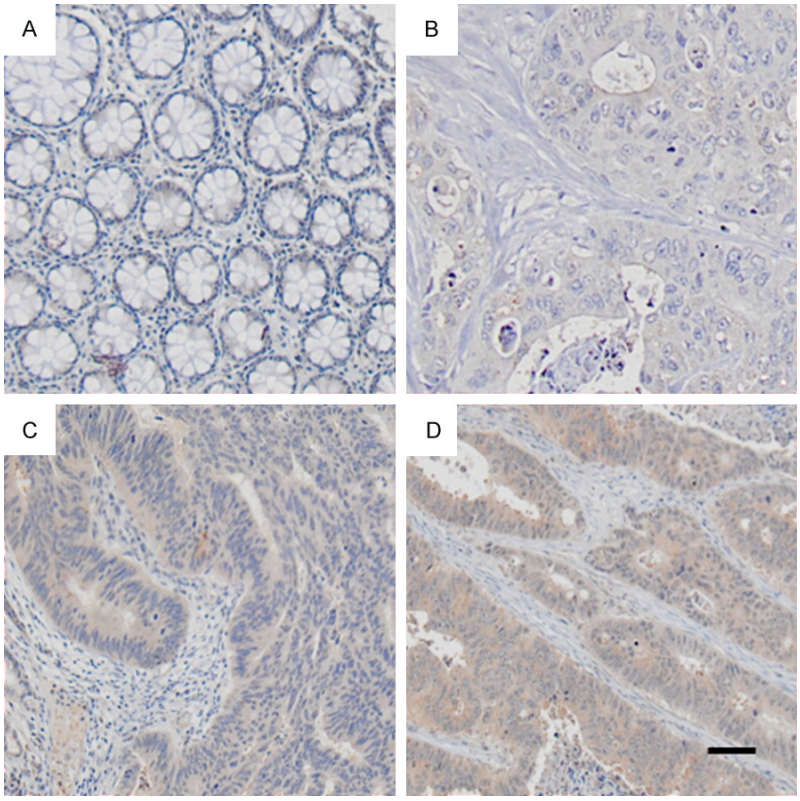

Figure 5. Immunostaining of eIF3C in CRC and tumor-adjacent tissues. A. Negative staining of eIF3C in tumor-adjacent tissues; B. Weakly positive; C. Positive; D. Strongly positive. Scale bar: 50 μm.

vitro data were statistically analyzed using SPSS 22.0 software by a paired t-test analysis method and prognosis data was analyzed by Cox regression. $P < 0.05$ was considered as statistically significant. Finally, the GraphPad Prism 5 and Adobe Photoshop CS5 were used to sketch the results.

Results

eIF3C mRNA expression levels in different CRC cell lines and infection of RKO cells

eIF3C expression levels in different CRC cell lines were first examined. RKO cells with a relative high eIF3C expression level were selected for the further research (**Figure 1A**). The eIF3C cells were successfully infected, and confirmed by a microscopic detection of green fluorescence. And the infection rate of RKO cells was more than 80% (**Figure 1B**).

eIF3C siRNA infection down-regulated eIF3C expression in RKO cells in both mRNA and protein levels

Through the RT-PCR experiment, mRNA level of eIF3C shRNA infected cells (Lv-eIF3C shRNA) was down regulated by 70% compared with control shRNA infected cells (Lv-Con) (**Figure 2A**). The protein level of Lv-eIF3C group was also obviously lower than the Lv-Con group (**Figure 2B**). We thus conclude that the expression level of eIF3C in RKO cells was suppressed by shRNA infection.

eIF3C shRNA infection suppressed the cell proliferation, colony formation, and affected the cell cycle of RKO cells

The cell proliferation rate decreased significantly from the third day growth. And after five days of growth, the number of cells in the

Table 1. Relative expression level of eIF3C with clinicopathological features in colorectal cancer patients

Feature	Total number of patients, n = 198	Number of patients		*P* value
		eLF3C low expression	eLF3C high expression	
Age (years)				0.694
<60	59	17	42	
≥60	139	44	95	
Gender				0.502
Male	98	28	70	
Female	100	33	67	
Location				0.800
Colon	142	43	99	
Rectal	38	18	38	
Tumor grade				0.035[a]
G1/G2	87	20	67	
G3/G4	111	41	70	
Size (cm)				0.006[a]
<5	87	18	69	
≥5	111	43	68	
Tumor invasion				0.458
T1/T2	35	10	25	
T3/T4	163	51	112	
Lymph node metastases				0.023[a]
Absent	105	25	80	
Present	93	36	57	
Distant metastasis				0.513
Absent	174	55	119	
Present	24	6	18	
TNM stage				0.077
I/II	90	22	68	
III/IV	108	39	69	

[a], Statistically significant. eLF3C, eukaryotic translation initiation factor 3; TNM, tumor-node-metastasis.

Lv-Con group was 60 times higher than Lv-eIF3C shRNA group (**Figure 3A**). The number of the colonies decreased significantly ($P < 0.01$) with the eIF3C deletion, showing a 3.5-fold decrease compared to the control group, and the size of each colony was smaller in Lv-eIF3C shRNA group (**Figure 3B**), indicating that the colony formation of the eIF3C shRNA infected cells was also suppressed. Because of the absence of the eIF3C gene, the number of colonies decreased. The Lv-eIF3C shRNA cells showed an 8% increase in the cells in G0/G1 phases ($P < 0.001$) and a 5% increase in G2/M phases, while the S phase decreased by 12% ($P < 0.001$) with the eIF3C deletion (**Figure 3C**). These results indicated that deletion of eIF3C had a significant influence on the survival and biological behavior of RKO cells.

The available cores of the TMA and the staining situation

Five TMA were made, and each side's core number from 1-5 was 123, 120, 120, 123, and 120, respectively (**Figure 4**). There were 202 patients met the criteria in total. However, some of the cores were not available because of the incompleteness of cores. The incomplete cores were 4, 5, 5, 7, and 4 individually. The available patients information were 40/41, 40/4040/40, 38/41, 40/40 (available patients number/total patients number). After the immunohistochemical staining, there were 202 cases in total. Taking into the account of the number of the incomplete cores, we excluded 4 cases. Finally, there were 198 cases available for the research, with 10 cases lost to follow up.

Unusual overexpression of eIF3C in CRC tissues

Immunostaining was performed to investigate differences of eIF3C protein expression between CRC and adjacent noncancerous tissues. As shown in **Figure 5**, eIF3C staining was almost negative in the adjacent noncancerous tissue (**Figure 5A**), whereas positive eIF3C staining with cytoplasm location was observed in CRC tissues (**Figure 5B-D**). eIF3C expression was detected in 69.19% (137/198) of the CRC samples, whereas almost all the noncancerous specimens exhibited a negative eIF3C signal ($P < 0.05$).

Relationship of eIF3C expression with clinicopathological features in CRC

The information of the patients used in this study was shown in **Table 1**. As we concluded, positive expression of eIF3C group was different from the negative group in some clinicopathological features, like tumor grade, tumor size, Lymph node metastases ($P < 0.05$). However, no statistical significance was found the about TNM stage ($P = 0.077$). The other fea-

Table 2. The data of overall survival and recurrence-free survival of colorectal cancer patients with different clinicopathological features

Variable	Overall survival			Recurrence-free survival		
	HR	95% CI	P	HR	95% CI	P
Tumor grade (G1/G2 vs. G3/G4)	2.470	1.342-4.544	0.004[a]	3.282	1.697-6.350	0.000[a]
Tumor invasion	1.982	0.6935.669	0.202	1.966	0.682-5.665	0.210
Tumor size (cm) (<5 vs. ≥5)	1.172	0.5942.312	0.647	1.240	0.6022.555	0.559
Lymph node metastases (absent vs. present)	0.817	0.2952.265	0.698	1.418	0.382-5.264	0.602
TNM stage (I/II vs. III/IV)	4.144	1.331-12.905	0.014[a]	1.794	0.465-6.922	0.397
eLF3C expression (low vs. high)	3.303	1.6096.781	0.001[a]	4.589	2.101-10.021	0.000[a]

[a], Statistically significant. eLF3C, eukaryotic translation initiation factor 3; TNM, tumor-node-metastasis; HR, hazard ratio; CI, confidence interval.

tures were similar, for instances, age, gender, location, tumor invasion, distant metastasis ($P > 0.05$).

Prognostic value of eIF3C expression in CRC patients

The prognostic value of eIF3C was analyzed by multivariate Cox regression analysis using SPSS. The tumor grade and eIF3C expression were closely associated with the overall survival (OS) and recurrence free survival (RFS), as **Table 2** showed. The TNM staging was also associated with OS. However, RFS was not influenced by it.

Discussion

The prognosis of CRC normally is related to histological grade, tumor size and completeness of excision. However, the TNM staging observed in clinic suggested that it might not be an exact independent prognostic factor for survival [7]. Some CRC patients with a huge tumor have a better prognosis than these with a tiny tumor. This may relate to the heterogeneity of colorectal cancer, but the TNM staging system failed to recognize it, which can cause the wrong treatment of CRC patients.

Translation and transcription levels now are taking a role in gene expression [8]. In eukaryotes, the regulation of translation initiation was mainly by 5' untranslated region (5' UTR), which can repress the expression of the downstream genes in a particular time and space [9]. The 5'UTR of highly expressed genes are usually short and simple, such as housekeeping genes, and can regulate a wide range of different cells but not inaccurately. Nevertheless, the specific regulation of some low expressed genes are always complicated with exact function, such as the genes of the growth factor and regulatory protein of cell proliferation, differentiation or apoptosis [10]. Most cancer cell translation initiation factors, such as eIF4E, eIF4G, eIF2, are overexpressed, which makes the inhibitory effect of 5'UTR weakened or even be neglected, so that the mRNA can translate excrescent proteins, such as the cell cycle regulatory elements and the products of the proto-oncogenes. For CRC, eIFs regulated 5'UTR was complicated, the abnormal regulation of these mRNA translation leads to the colorectal cells malignancy and causes the colorectal cancer [11]. Therefore, the expression of eIFs could be used to predict the survival and metastasis, and some of them, like eIF4E, becomes a potential therapy target in CRC [12].

eIF3 might be exploited as a tumor marker for clinical application [13]. It was reported that eIF3's subunits, including eIF3A, eIF3E, eIF3D, eIF3F and eIF3I, are often aberrant in many tumors and closely related to initiation, invasion, metastasis and prognosis of the malignancy. In addition, eIF3C is the kernel component of the core complex that keeps the activity of eIF3 and is obligatory to start the translation [14, 15], and mediated the interaction between eIF3 complex and other translation initiation factors [16]. It is important that the richness and assembly of the whole eIF3 under the thumb of eIF3C and eIF3a, hence on behalf of the key support elements needed to form the PICs [17]. From the gene analysis of 33 different strains of mice with colorectal cancer, the eIF3C was proven to be one of the most common mutant genes [18]. In many other research of eIF3C, it was proven to be influential [19]. However, there are very few researches about

eIF3C and colorectal cancer. Not only the mechanism but also the clinical evidence is absent.

Comparing the levels of eIF3C mRNA in five colorectal cancer cell lines (RK0, HCT116, SW480, SW620 and LoVo), the results showed that eIF3C mRNA was highly expressed in four of the five cancer cell lines (**Figure 1**). We found that the level of eIF3C mRNA decreased by 70% ($P < 0.001$) and the protein level of eIF3c also declined obviously after interfered with eIF3c-shRNA interference system. The rate of cell proliferation was obviously lower in interference group than that of the control group (1:60, $P < 0.01$); the deletion of eIF3c gene significantly reduced the colony size and decreased cell colony formation by 35% comparing with the control group ($P < 0.01$). Flow cytometry analysis revealed that after the deletion of eIF3c gene, G0/G1 phase cells increased by 8% ($P < 0.001$) and G2/M phase cells increased by 5% ($P < 0.01$), while S phase of the cells decreased by 12% ($P < 0.001$). Taken these results together, eIF3C can affect tumor proliferation, colony formation and cell cycle.

Several studies have shown that eIF3C is a potential therapeutic target for cancer [20]. To investigate the effects of eIF3C to the CRC patient prognosis, we used TMAs and immunohistochemistry to predict prognostic. TMA has the considerable effect of drastically diminishing costs and other resources as well as reducing tissue wasting. Additionally, in this research, we compared the clinical features of the highly expressed eIF3C weakly expressed group. The eIF3C strongly expressed group was different from the weakly expressed group in terms of the tumor grade, tumor size, lymph node metastases ($P < 0.05$). However, no statistical significance was found the about TNM stage ($P = 0.077$). However, we can see that $p = 0.077$ is statistic significant. Maybe with more patients and longer follow-up time, we can further confirm the finding. Through the COX regression model analysis, some interesting phenomena were observed. TNM staging could predict the OS, but couldn't predict the RFS; eIF3C expression level and pathological staging are related more with OS and thus are able to be used predict the RFS. The result suggested l that eIF3C expression level can be employed to prognosis more precisely than the TNM staging.

In conclusion, through *in vitro* experiments and clinical studies, we found that eIF3C is highly expressed in CRC cells and can influence the tumor proliferation, colony formation and cell cycles. In addition, eIF3C is an independent risk factor for CRC patients both OR and RFS. By synthesis of TNM staging, pathological staging and eIF3C expression level, physicians could make a more precise treatment and follow-up schedule for the patients specifically. However, the current research may need to be confirmed further using a large clinical sample size.

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Disclosure of conflict of interest

None.

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