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Original Article

eIF3d is an independent prognostic parameter in colorectal cancer and promotes proliferation via c-Jun/cyclin D1 in CRC cell line

Chengang Yang[1], Bingbo Zhao[1], Chaobin Li[2]

Departments of [1]Gastrointestinal Surgery, [2]Gastroenterology, Liaocheng People's Hospital, School of Taishan Medical University, Liaocheng, Shandong Province, China

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Abstract: The eIF3d is overexpressed in many different kinds of cancer cells. In our study, we showed that eIF3d was significantly up-regulated and associated with tumor size, clinical stage, and metastasis in CRC patients. Additionally, the expression of eIF3d was correlated with the prognosis of CRC. Furthermore, we demonstrated that knockdown of eIF3d inhibited cell proliferation and reduced c-Jun and cyclin D1 expression in CRC cells. Taken together, we revealed that eIF3d could activate c-Jun/cyclin D1 pathway, which is involved in cell cycle and proliferation of CRC cells. Based on our findings, eIF3d might act as an independent prognostic factor and therapeutic target for the treatment of human CRC.

Keywords: eIF3d, c-Jun, cyclin D1, proliferation, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death worldwide [1]. The basic therapy including surgery, neoadjuvant radiotherapy, and adjuvant chemotherapy. However, the five-year relative survival still low in stage IV CRC patients [2]. Therefore, novel biomarkers for therapeutic approaches preventing cancer cell progression is highlighted for CRC treatments. eIF3d (eukaryotic translation initiation factor 3 subunit D), a member of the eIF3 family, is an mRNA cap-binding protein [3]. The increased expression of eIF3d gene are found in many different kinds of cancer cells [4-10]. The silencing of eIF3d results in decreased proliferation, clonality and invasiveness in prostate cancer cells [4]. Knockdown of eIF3d inhibits cell proliferation in non-small cell lung cancer [6]. Recently, eIF3d has been found to be essential for assembly of translation initiation complexes on eIF3-specialized mRNAs such as the cell proliferation regulator c-Jun [11].

In the present study, we evaluated eIF3d expression in CRC tissues and the correlation between eIF3d expression and clinicopathological characteristics or prognosis of CRC patients. Furthermore, we revealed that eIF3d promoted CRC cell proliferation via c-Jun/cyclin D1 pathway.

Material and methods

Tissue collection and ethics statement

A total of 93 paired tumor tissues and matched normal tissues (> 2.0 cm distance from the tumor edge) were collected from patients with colorectal cancer (CRC) who received surgical treatment between August 2012 and September 2016 at The Liaocheng People's Hospital, School of Taishan Medical University (Shandong, China). All experiments were approved by the Research Ethics Committee of Taishan Medical University (Jiaxing, China). Written informed consent was obtained from all patients. All patients signed their written informed consent to participate in this study. Fresh-frozen samples were used for eIF3d expression analysis.

Cell culture

The human colorectal cancer cell lines SW480 and HT-29 were cultured in DMEM Medium (Life

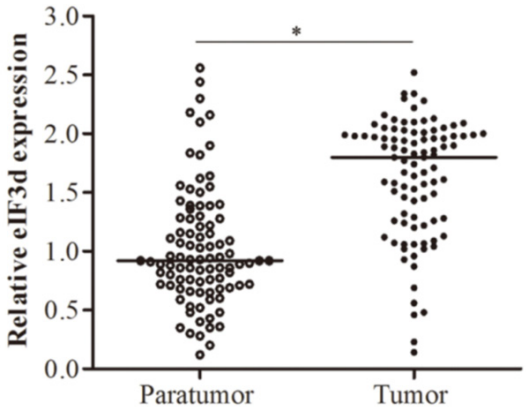

Figure 1. Expression of eIF3d in CRC cases. Representative real-time PCR analysis of eIF3d expression in CRC tumor tissues and matched paratumor tissues was shown. n = 93; Values are depicted as median; *$P < 0.05$ by t test.

Technologies, USA) supplemented with 10% FBS (Life Technologies, USA) in a humidified 5% CO_2 atmosphere at 37°C.

siRNA transfection

Transfection experiments were performed by using Lipofectamine 2000 kit (Invitrogen, USA). The siRNA sequence targeting EIF3D was 5'-GCGTCATTGACATCTGCATGA-3'. Short interfering RNA targeting eIF3d (Santa Cruz Biotechnology, Santa Cruz, USA) was transfected into cells using Lipofectamine 2000 reagent according to the manufacturer's instructions.

RNA isolation and real-time PCR

RNA was isolated from tissues or cells using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized from total RNA using PrimeScript RT-PCR Kit (TAKARA, Japan). Then, real-time PCR was run on a StepOne Plus™ real-time PCR instruments (Applied Biosystems, USA). Semi-quantitative real-time PCR using SYBR Green (Takara, Japan) was performed to compare the relative expression levels of eIF3d mRNA according to the manufacturer's instructions.

Western blotting

Whole cells were washed in PBS and lysed in RIPA lysis buffer (Beyotime, China) supplemented with protease inhibitor cocktail (Roche, Germany). Total protein was quantified using a BCA Protein Assay Kit (Beyotime, China), and an equal amounts of whole cell lysates was resolved by 10% SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 5% BSA (5% w/v in PBS + 0.1% Tween 20) and incubated with primary antibodies at room temperature. The antibodies which are against eIF3d (ab155419), c-Jun (ab218576), cyclin D1 (ab134175) and GAPDH (ab8245) were used according to the manufacturer's instructions, and were purchased from Abcam (Cambridge, USA). The appropriate secondary antibodies (Santa Cruz, USA) were used at 1:2,000-1:5,000 (v/v) dilutions in PBS + 0.1% Tween 20 for 1 h at room temperature, and the signals were revealed using ECL kit (Thermo Scientific, USA).

Cell proliferation and cell cycle assay

Cell proliferation was performed with Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). According to the instructions, Cell Counting Kit-8 reagent was added at 0, 24, 48, and 72 h respectively after seeding 5×10^3 cells per well in a 96-well plate and incubated at 37°C for 2 h. The OD (optical density) 450 nm value was detected by using a microplate reader (Bio-Rad, USA). Cell cycle assay was evaluated with an PI/RNase Staining kit (BD Pharmingen, USA) according to the manufacturer's instructions. The flow cytometry with a FACS Calibur (BD Bioscience, USA) was performed to evaluate the result.

Statistical analysis

All results were expressed as the mean ± s.d. The Student's t-tests were used to analyze significant differences between samples. Chi-square tests were used to examine the association between the clinicopathological characteristics and eIF3d expression. In addition, univariate and multivariate survival analyses were conducted by using the Cox proportional hazards model. The Kaplan-Meier method was used to compare overall survival and the log-rank test was used to evaluate differences in survival. All the histogram was evaluated by performing GraphPad Prism, version 4.0 (GraphPad Software, USA). Statistical analyses were performed using Stata. 11.0. $P < 0.05$ indicated statistically significant.

Table 1. Correlation between eIF3d expression and clinicopathological characteristics of colorectal cancer patients (n = 93)

Characteristics	All Patients	eIF3d low expression (< Median[a])	eIF3d high expression (≥ Median[a])	*P* value Chi-squared test
No.	93	46 (49.5%)	47 (50.5%)	
Age (years)				0.934
< 60	36	18 (50%)	18 (50%)	
≥ 60	57	28 (49.1%)	29 (50.9%)	
Gender				0.608
Male	49	23 (46.9%)	26 (53.1%)	
Female	44	23 (52.3%)	21 (47.7%)	
Histology				0.610
AC	59	28 (47.5%)	31 (42.5%)	
MC	34	18 (52.9%)	16 (47.1%)	
Tumor size				0.040*
T1/T2	31	20 (64.5%)	11 (35.5%)	
T3/T4	62	26 (41.9%)	36 (58.1%)	
Clinical stage				0.005*
I-II	39	26 (66.7%)	13 (33.3%)	
III-IV	54	20 (37.0%)	34 (63.0%)	
Metastasis				0.030*
Yes	25	17 (68.0%)	8 (32.0%)	
No	68	29 (42.6%)	39 (57.4%)	

[a]The median expression level of eIF3d was used as the cut-off. *Indicates *P* value < 0.05. AC: Common adenocarcinoma. MC: Mucinous adenocarcinoma.

Results

Increased eIF3d expression was significantly associated with tumor size, clinical stage, and metastasis in colorectal cancer patients

We examined the expression levels of eIF3d in 93 colorectal cancer (CRC) clinical samples via utilizing real-time PCR, with quantified values used to calculate eIF3d/GAPDH ratios. Our results suggested that the relative expression levels in tumor tissues were significantly higher compared with those in paratumor tissues (**Figure 1**). Based on the median value of eIF3d expression levels, we divided the 93 CRC patients into two groups: eIF3d high-expression group (47 cases) and eIF3d low-expression group (46 cases). Then, we performed Chi-square tests to explore the association between the clinicopathological characteristics and eIF3d expression. The findings were summarized in **Table 1**. Increased eIF3d expression was significantly associated with tumor size (*P* = 0.040), clinical stage (*P* = 0.005), and metastasis (*P* = 0.030). No significant correlations were found between eIF3d expression and age, gender or histology.

The prognostic significance of eIF3d in CRC

We next explored whether eIF3d expression correlated with the prognosis of CRC. We performed an analysis of 3-year overall survival and relapse-free survival. Our findings indicated that the high eIF3d expression group had a significantly poorer prognosis compared with the low expression group (**Figure 2**). Additionally, in the univariate Cox hazard regression analysis, the high eIF3d expression group attained an obviously higher overall mortality compared with the low eIF3d expression group (hazard ratio, 2.272; 95% confidence interval, 1.099-4.695; P ¼, 0.027; **Table 2**). In the multivariate Cox hazard regression analysis for overall survival including age at operation, N status, venous invasion, and eIF3d expression, high eIF3d expression was revealed to be an independent prognostic factor (multivariate hazard ratio, 2.425; 95% confidence interval, 1.205-4.878; P ¼, 0.013; **Table 2**).

Knockdown of eIF3d inhibited cell proliferation in CRC cells

The significant correlation between eIF3d expression and clinicopathological characteristics suggested that eIF3d might act a vital role in the development and progression of CRC. Based on the expression pattern of eIF3d, the influences of overexpression of eIF3d on cell proliferation were examined in human CRC cell lines. The CRC cell lines SW480 and HT-29 were transfected with scramble and siRNA-eIF3d. As shown in **Figure 3A** and **3B**, real-time PCR and western blotting were performed to test the mRNA and protein expression levels of

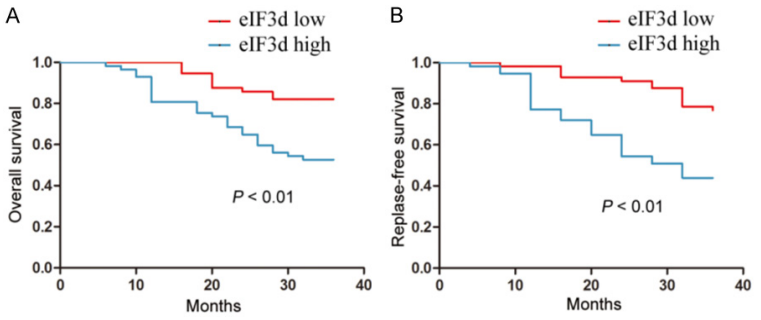

Figure 2. The prognostic significance of eIF3d in CRC. (A) Kaplan-Meier overall survival and (B) relapse-free survival of patients with CRC according to eIF3d expression. The overall survival and relapse-free survival rate of patients with tumors that were assigned to the high-eIF3d-expression group was significantly lower than that of patients with tumors that were assigned to the low-eIF3d-expression group.

Table 2. Cox proportional hazards analysis of survival for CRC

A.			
Clinical factor	*P* value	Univariable analysis	
		HR	95% CI
eIF3d			
< Median[a] *vs* > Median[a]	0.002	3.230	1.562-6.679
Age			
< 60 *vs* ≥ 60	0.541	1.223	0.641-2.330
Gender			
Male *vs* Female	0.911	1.039	0.534-2.019
Histology			
AC *vs* MC	0.923	0.968	0.505-1.856
Tumor size			
T1+T2 *vs* T3+T4	0.013	2.271	1.191-4.330
Clinical stage			
I+II *vs* III+IV	0.000	12.955	4.576-36.674
Metastasis			
No *vs* yes	0.021	1.895	1.133-2.690
B.			
Clinical factor	*P* value	Multivariable analysis	
		HR	95% CI
eIF3d			
< Median[a] *vs* > Median[a]	0.001	5.871	2.349-14.670
Age			
< 60 *vs* ≥ 60	0.864	0.932	0.418-2.077
Gender			
Male *vs* Female	0.517	1.250	0.636-2.458
Histology			
AC *vs* MC	0.296	1.471	0.714-3.033
Tumor size			
T1+T2 *vs* T3+T4	0.037	2.222	1.047-4.713
Clinical stage			
I+II *vs* III+IV	0.000	14.208	4.804-42.017
Metastasis			
No *vs* yes	0.007	3.147	1.372-7.219

P < 0.05 indicated statistically significant. [a]The median expression level of eIF3d was used as the cut-off. HR: Hazard ratio. CI: Confidence interval.

eIF3d. The mRNA and protein levels of eIF3d were significantly reduced following transfection of siRNA-eIF3d in the SW480 and HT-29 cells, respectively, comparing with the scramble groups. These results suggested that the expression of EIF3D was significantly downregulated after siRNA-eIF3d transfection. Besides, cell proliferation was examined following eIF3d knockdown using an CCK-8 assay. The results demonstrated that down-regulation of eIF3d inhibited the proliferation ability compared with scramble in CRC cells (**Figure 3C**). Taken together, our findings verified that knockdown of eIF3d inhibited cell proliferation in CRC cells.

Knockdown of eIF3d induced cell cycle arrest via inhibiting c-Jun/cyclin D1 pathway

To investigate the effects of eIF3d downregulation on cell cycle, SW480 and HT-29 cell lines with low eIF3d expression were stained with propidium iodide and analyzed by flow cytometry. As shown in **Figure 4A**, knockdown of eIF3d in SW480 and HT-29 significantly increased the cell population in G1 phase and reduced the cell population in S phase, indicating that knockdown of eIF3d results in G1/S phase arrest. Taken together, eIF3d knockdown in colorectal cancer cells delays cell cycle progression, thereby influencing the cell growth. Furthermore, we confirmed decreased expression of c-Jun and cyclin D1 by western blotting in SW480 and HT-29 cell lines with low eIF3d expression (**Figure 4B**). In additional, cyclin D1 is regulated by c-Jun during the cell cycle. Thus, knockdown of eIF3d in-

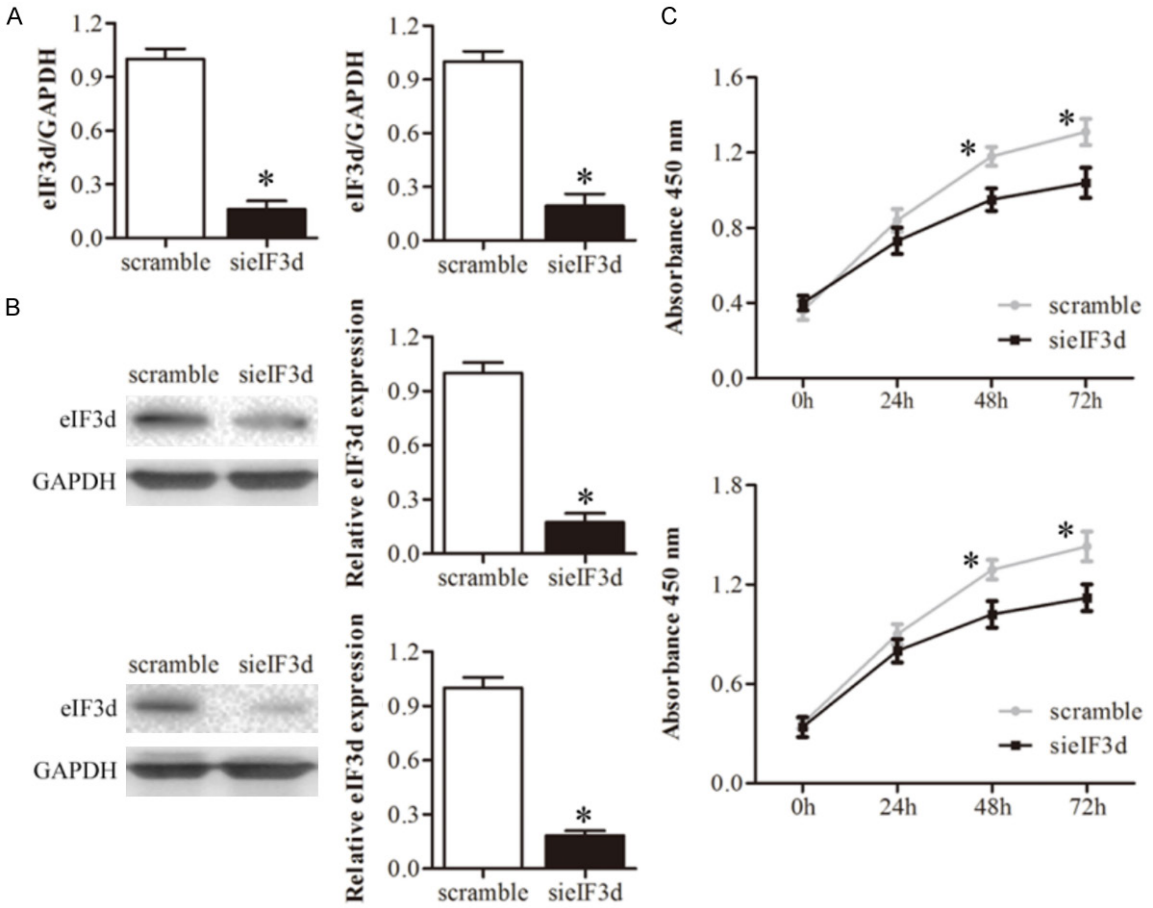

Figure 3. Knockdown of eIF3d inhibited cell proliferation in CRC cells. (A) and (B) CRC cells SW480 and HT-29 were transfected with scramble or siRNA-eIF3d. Twenty-four hours later, the expression levels of eIF3d mRNA and protein were evaluated using real-time PCR and Western blotting, respectively. The results obtained from three independent experiments were presented as the mean ± s.d. *$P < 0.05$ by t test. (C) CCK-8 proliferation assay was performed using scramble or siRNA-eIF3d transfected CRC cells SW480 and HT-29. Absorbance at 450 nm was presented with mean ± s.d. The results obtained from three independent experiments were presented as the mean ± s.d. *$P < 0.05$ by t test.

duced cell cycle arrest in colorectal cancer cells, probably mediated via inhibiting c-Jun/cyclin D1 pathway.

Discussion

Our present study suggested that eIF3d was also obviously up-regulated in human colorectal cancer tumor tissues. Additionally, the overexpression of eIF3d correlated with the poor prognosis of CRC patients. Several reports have suggested that the inhibition of cell cycle progression may be an appropriate target for the management of cancer [12]. In our study, silencing of eIF3d inhibited cell proliferation in CRC cells. Importantly, eIF3d could promote the translation initiation of c-Jun mRNA [11]. The proto-oncoprotein c-Jun belongs to the dimeric AP-1 family of transcription factors, which are important regulators of cellular proliferation, apoptosis and tumorigenesis. [13]. Furthermore, c-Jun has been implicated in the tumorigenesis of colon epithelium [14].

Recent study has shown that eIF3d enhances c-Jun level via protecting the 5' end of c-Jun mRNA [3]. Therefore, we aimed to identify whether knockdown of eIF3d could induce G1/S arrest in CRC cells. Interestingly, we found that the silencing of eIF3d in CRC cells delayed cell cycle progression, thus influencing the cell growth. The cyclin D1 is a critical regulator of the transition from G1 to S phase and may serve to increase oncogene expression via its

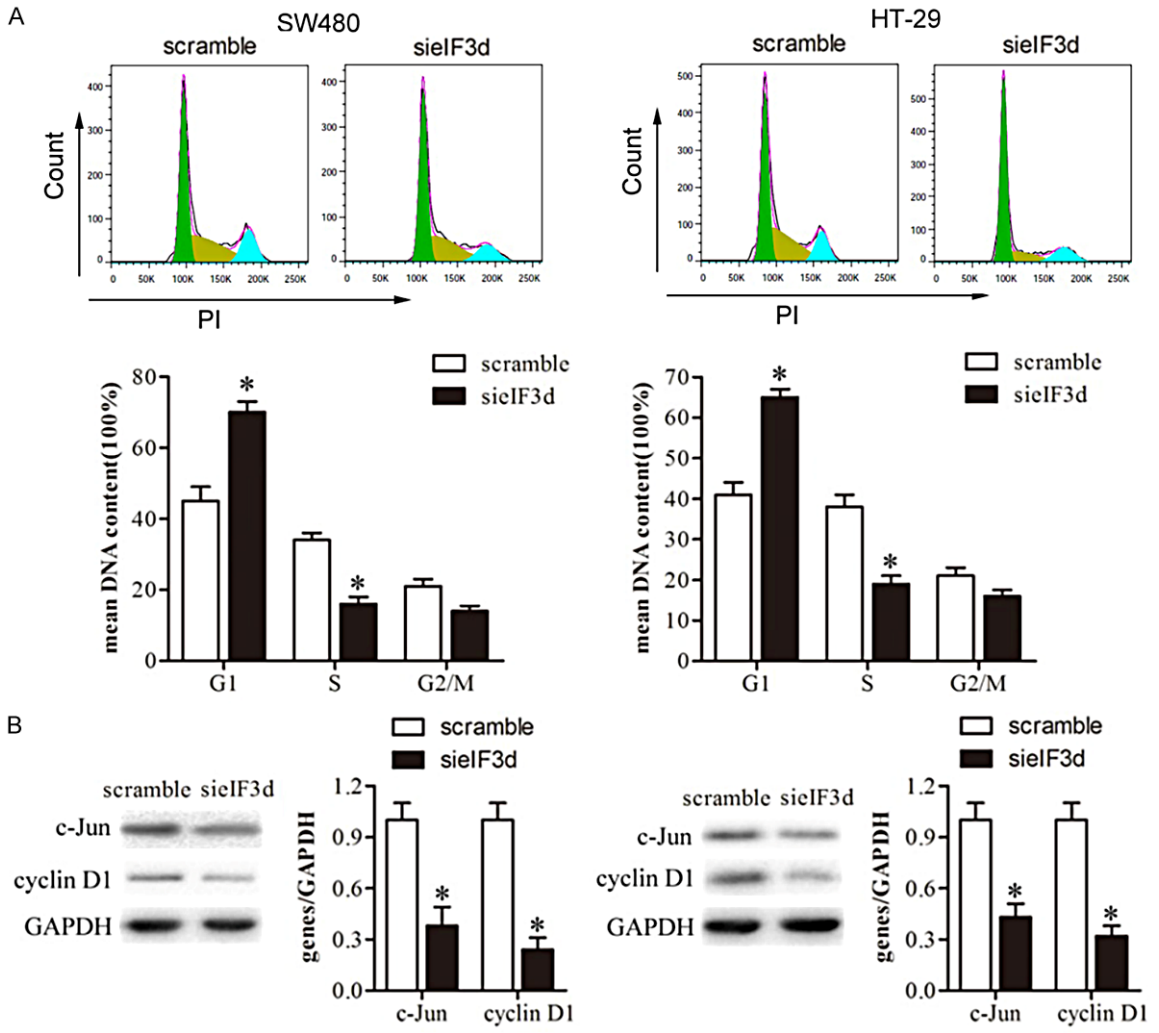

Figure 4. Knockdown of eIF3d induced cell cycle arrest via c-Jun/cyclin D1 pathway. A. Cell cycle analysis with flow cytometry. CRC cells SW480 and HT-29 were transfected with scramble or siRNA-eIF3d. Twenty-four hours later, cells were stained with propidium iodide and analyzed. The results obtained from three independent experiments were presented as the mean ± s.d. *$P < 0.05$ by t test. B. c-Jun and cyclin D1 protein levels were analyzed by Western blotting in scramble or siRNA-eIF3d transfected SW480 and HT-29 cells. The results obtained from three independent experiments were presented as the mean ± s.d. *$P < 0.05$ by t test.

cell-cycle regulating function [15]. Moreover, cyclin D1 is significant prognostic marker in colorectal cancer patients and promotes the growth and tumorigenesis of human colon cancer cells [15, 16]. Importantly, the activity of cyclin D1 promoter was shown to be directly regulated by c-Jun [16]. To further confirm that knockdown of eIF3d induced cell cycle arrest is partly dependent on c-Jun/cyclin D1 pathway, we performed western blotting to confirm the effects of eIF3d knockdown on c-Jun and cyclin D1 protein levels. Our results indicated that the expression of c-Jun and cyclin D1 was decreased in CRC cells with knockdown eIF3d expression.

Taken together, we demonstrated that eIF3d promoted cell proliferation via c-Jun/cyclin D1 pathway in CRC cells.

Based on our findings, eIF3d might act as an independent prognostic factor and therapeutic target for the treatment of human CRC.

Disclosure of conflict of interest

None.

Address correspondence to: Chengang Yang, Department of Gastrointestinal Surgery, Liaocheng People's Hospital, School of Taishan Medical University, No. 67 Xichangxi Road, Liaocheng

252000, Shandong Province, China. Tel: +86 0635-8276110; Fax: +86 0635-8966225; E-mail: bakerham123@163.com

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