

## Original Article

# FL118 inhibited the proliferation of colorectal carcinoma cells via upregulating CDC73

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**Abstract:** Objective: Camptothecin and its derivatives are broad-spectrum anti-cancer drugs which commonly used in the clinical treatment. FL118 is a new generation of camptothecin analogue. Previous studies have reported that FL118 exerts higher antitumor activity than SN-38, and even, some first-line chemotherapeutic agents. However, the underlying molecular mechanism of FL118's anti-tumor activity is still unclear. Methods: MTT assay, scratch wound assay and BrdU cell proliferation assay were employed to test colorectal carcinoma cell viability, mobility and proliferation under the treatment of FL118. We also observed the effect of FL118 on tumor inhibition in xenograft models. Immunoblotting and qPCR were further performed to detect the expression of CDC73 in colon cancer cells and tumors after treated with FL118. Then, we detected the expression of CDC73 in the human colorectal cancer and adjacent tissues. Results: FL118 remarkably inhibited cell viability, migration and proliferation of colon cells *in vitro* and suppressed the growth of tumor *in vivo*. The expression of CDC73 was lower in colorectal cancer than adjacent tissues. FL118 significantly upregulated the expression of CDC73 both *in vivo* and *in vitro* ( $P < 0.05$ ), and negatively regulated Wnt/ $\beta$ -catenin pathway. Conclusions: FL118 can effectively inhibit the growth of colorectal cancer and upregulate the expression of CDC73 *in vivo* and *in vitro*. CDC73 may act as a tumor suppressor gene in colorectal cancer and mediate antitumor effect of FL118 by affecting Wnt/ $\beta$ -catenin pathway.

**Keywords:** FL118, CDC73, colorectal carcinoma cells, proliferation

## Introduction

Colorectal cancer (CRC) is a common malignant gastrointestinal tumor with high morbidity and mortality among digestive system tumors [1]. It is well known that Camptothecin and its derivative are used as common drugs for cancer therapy. For example, irinotecan (CPT-11), a camptothecin derivative approved by Food and Drug Administration (FDA), is applied for advanced CRC treatment [2].

Recently, a novel camptothecin analogue, named FL118, was selected from small molecule libraries for its high activity as a survivin gene inhibitor. However, studies have found that FL118 could also selectively inhibit other apoptosis-associated members, including XIAP, cIAP2, Bcl-2 and Mcl-1 [3]. Although the molecular structure of FL118 is similar to topotecan and irinotecan [4, 5], yet their anticancer capability is of distinct difference. Moreover, FL118

was reported to effectively eliminate xenograft tumor *in vivo* at lower dose with lower toxicity compared with topotecan and irinotecan. Importantly, FL118 was demonstrated to exert superior effect on large ( $\sim 2000$  mg/mm<sup>3</sup>) and advanced tumors [6]. Interestingly, studies have shown that FL118 failed to significantly inhibit the activity of TOP1 (DNA topoisomerase 1), a known target of SN-38 (the active ingredient of irinotecan) and other camptothecin derivative, indicating other important molecular mechanisms underlying FL118's excellent anti-tumor activity.

CDC73 (Cell Division Cycle 73), also named HRPT2 (hereditary hyperparathyroidism type 2), was mapped to 1q21-q32 [7]. The 531-amino acid parafibromin encoded by CDC73 forms polymerase-associated factor1 (Paf1) complex [8, 9]. Which regulates whole gene expression, and is involved in transcriptional and posttranscriptional events [10, 11]. It has been widely

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detected in various tissues [12], such as kidney, adrenal gland, parathyroid gland, heart, and skeletal muscles. Researchers have found that CDC73 inhibited the cell proliferation and induced cell cycle arrest in HeLa cells [13]. Furthermore, overexpression of CDC73 can decrease  $\beta$ -catenin mediated transcription in human colorectal cancer cells and B cells [14]. These findings suggest that CDC73 plays an important role in the development and progression of tumors.

In this study, we examined the antitumor activity of FL118 in CRC *in vitro* and *in vivo* and found that FL118 could significantly inhibit the cell viability, proliferation and migration of CRC cells. Besides, the expression of CDC73 and  $\beta$ -catenin after FL118 treatment were also detected by qRT-PCR and western blot. Moreover, we employed siRNA interference to knock-down CDC73 to clarify the relationship between CDC73 expression and FL118's antitumor activity.

### Materials and methods

#### *Cell line and reagents*

The human colorectal cancer cells HCT-116, LOVO, SW480 were kindly provided by Institute for Translation Medicine of Qingdao University (Shandong, China). LOVO was cultured in RPMI-1640 with 10% FBS (PAN), HCT-116 and SW480 were cultured in DMEM supplement with 10% FBS (PAN). All cells were cultured in a 5% CO<sub>2</sub> atmosphere at 37°C. SN-38 (Selleckchem Chemical, China), FL118 was donated by Dr. Li Fengzhi laboratory of the United States (Roswell Park Cancer Institute, USA), they were prepared as stocks at 1 mM in DMSO (Sigma, China).

#### *Cell viability assay*

Cell viability was determined by MTT assay. Cells were prepared into single cell suspension and were seeded in 96-well plates (Corning, USA), FL118 was added to every sample at a series of concentrations (0.01, 0.1, 1, 10, 100 and 200 nM) after cell adherent. SN-38 (Positive control drug) and DMSO were 100 nM. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) act as a indicator to detect cell viability after different time: 24, 48, 72 h. Discard the culture medium and then add 150  $\mu$ l DMSO each well. The OD value was

measured by Multifunctional enzyme marker (Bio-Tek instruments) at 490 nm, and was calculated cell proliferation inhibition rate (IR) = 1 - (the OD of experimental group/the OD of control group).

#### *Wound healing assay*

HCT-116 cells were seeded into 6-well plate. Linear wound tracks were generated with 10  $\mu$ l micropipette tip, and discard the non-adherent cell and washed with PBS two times. The control group added 2 mL medium containing 1% FBS, the groups with different FL118 concentrations (0, 10 and 100 nM). The distance of cells migration at 0, 24 and 48 h after scratching was recorded, three independent scratch-wound experiments were used to be collected, cell migration rate = (distance at 0 hour - distance at 24/48 hours)/distance at 0 hour.

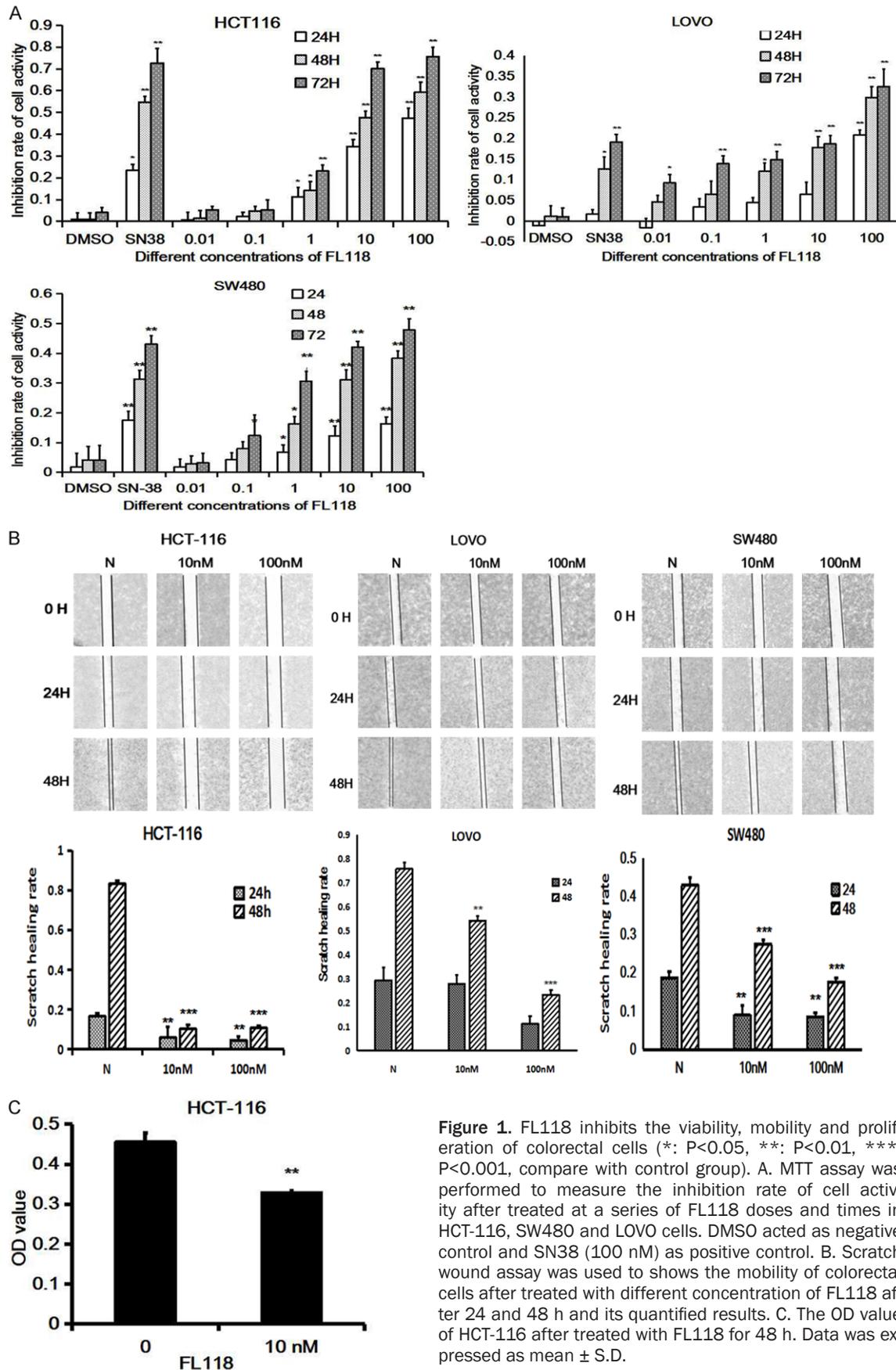
#### *Quantitative real-time PCR*

Total RNA was extracted from cell using TRIzol (CWBI). Total RNA (1  $\mu$ g per sample) was converted to cDNA using FastQuant RT Kit (with gDNase) (TIANGEN) following the manufacturer's instructions. Individual reverse transcription reactions in a total volume of 20  $\mu$ l, 3  $\mu$ l was used for real-time qPCR using the SuperReal PreMix Plus (SYBR Green) kit (TIANGEN). The sequences of CDC73 primers used in real-time PCR were: GAGAGAGTATGGAGGACACGAAC (forward), TTTGGGGCAGGTCGCTGTTCA (reverse).  $\beta$ -actin was used as an internal control, the primers of  $\beta$ -actin were AAGAGAGGCATCCT GACCCT (forward), TACATGGCTGGGGTGTGAA (reverse). Three samples were tested in each condition of control in parallel. The qRT-PCR condition is 95°C for 15 min, followed by 40 PCR cycle at 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec. The data were analyzed using Bio-Rad CFX manager.

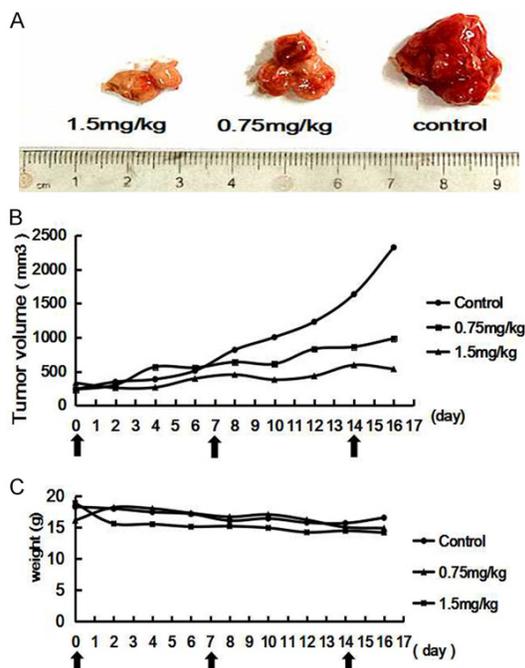
#### *Immunoblotting assay*

Proteins of human tissues or cultured cells were harvested with RIPA lysate (tissue/cell) Extraction Reagent (Solarbio, Beijing, China), separated using 15% polyacrylamide gel electrophoresis and transferred onto 0.45  $\mu$ m nitrocellulose membranes after activation with methanol. The membranes were blocked with 5% fat-free dry milk in phosphate-buffered

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**Figure 2.** The effect of FL118 in HCT-116 tumor bearing mice and the expression of CDC73, mice was treated with FL118 through intratumoral injection and the days of FL118 administration are annotated by bold arrow symbols. Totally, FL118 treatment was lasted for sixteen days. The institutional and national guide for the care and use of laboratory animals was followed. A. Solid image of tumor after Autopsy of nude mice. B. The size of the tumor after treated with different doses of FL118. C. The changes of body weight in different concentrations of FL118.

saline for 1 hour and incubated with primary antibodies at 4°C for one night, the membrane was incubated with corresponding secondary antibodies for 1 hour and visualized with high sensitivity chemiluminescence detection kit (CWBI0, Beijing, China).

### Patients tissue samples

Colorectal cancer and adjacent tissues were collected at the Departments of Surgery of the affiliated Hospital of Qingdao University of China. The study was approved by our Internal Review Board and informed consent was obtained where required.

### SiRNA transfection

Experimental operation procedure followed to TaqManfection reagent operating instructions. The logarithmic growth of HCT-116 cells were seeded in 6-well plate, then discard old medi-

um, siRNA and lipo RNAiMAX were added after diluted with an appropriate amount of serum free DMEM. The mixture added six well plate after 30 min in room temperature. After 4-6 hours, replace it with complete culture medium and the cells were cultured in incubator for 24 hours, and continue with other follow-up experiments.

### BrdU labeling cell proliferation assay

Cells were seeded in 96-well plate at  $2 \times 10^4$ /well, and were transfected with siRNA according to the transfection method. Cells were treated with FL118 at 10 nM for 48 hours, and  $1 \times$  BrdU was added into proliferating cells 2.5 hours ahead of schedule. Parallel wells with culture media only and cells plated without adding the BrdU Reagent should be prepared at the same time. Follow up the experimental procedures in accordance with BrdU Cell Proliferation ELISA Kit (colorimetric, Abcam).

### Animal experiments

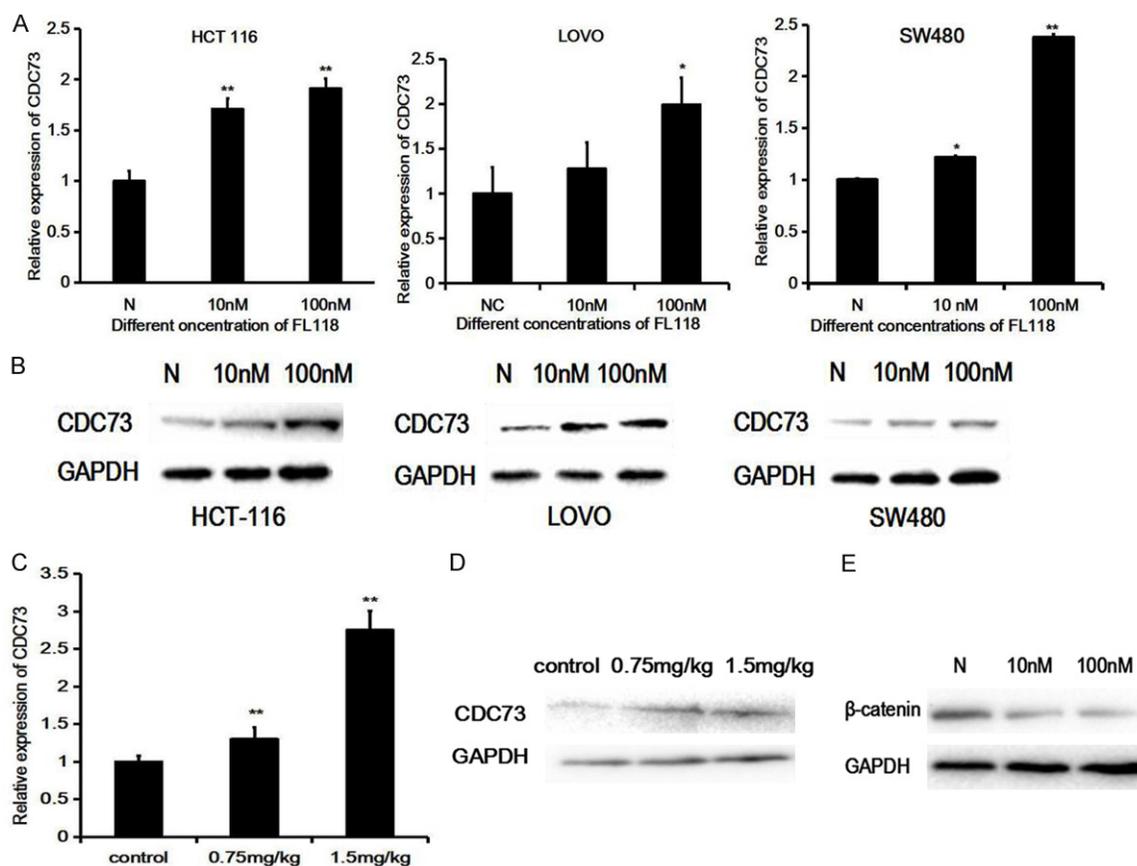
Seven to eight-week-old female (weight 15 to 22 g) purchased from Beijing Vital River Laboratory Animal Technology Corporation (China) to establish xenograft models. 100  $\mu$ l HCT-116 cells (about  $1-2 \times 10^6$ ) suspension was injected subcutaneously into nude mice, 3 nude mice with similar weight and tumor size were divided into one group until the size of the transplanted tumor was greater than 100 mm<sup>3</sup>.

There were three treatment conditions: control group (normal saline), FL118 low dose group (0.75 mg/kg) and high dose group (1.5 mg/kg). Intratumoral injection was used and the medication regimen referred to the clinical camptothecin analogues: the treatment schedule is one treatment per week for four weeks when HCT-116 xenografts volumes reached about 100 mm. The weight and volume of the transplanted tumor were measured ( $V = 0.5 \times (\text{length} \times \text{width})^2$ ) every another day during the treatment. When the nude mice were in a dying state or the volume of the transplanted tumor was greater than 1500 mm<sup>3</sup>, the experiment was finished.

### Statistical analysis

All experiments and measurements were performed for at least three times. Statistical anal-

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**Figure 3.** FL118 could up-regulate CDC73 and inhibited  $\beta$ -catenin. (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ , compare with control group). HCT-116, SW480 and LOVO cells were treated with different concentration of FL118 (0, 10, 100 nM) for 48 h. A and B. Relative expression of CDC73 mRNA and protein in HCT116, SW480 and LOVO cells in vitro. C and D. The up-regulation of CDC73 in FL118-treated tumor samples compared to control group in mRNA and protein level. E. The effect of FL118 on the protein level of  $\beta$ -catenin.

yses were performed using student's t-test. Data were considered to be statistically significant when  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*).

### Results

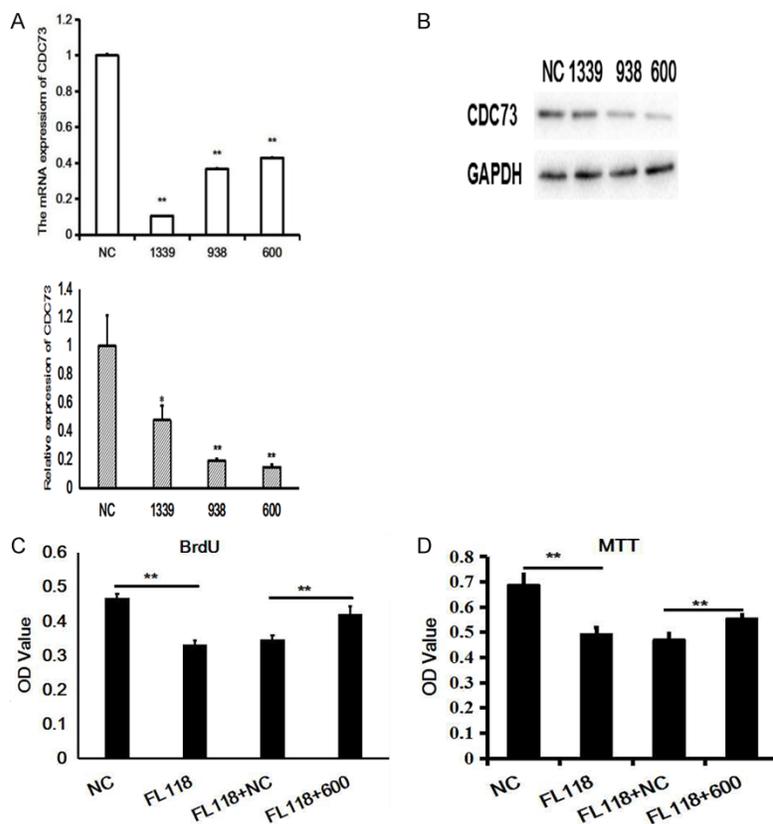
#### FL118 significantly inhibited multiple malignant phenotype in CRC cell lines

MTT assay was performed to detect the cell viability. HCT116, LOVO, SW480 cells were treated with FL118 at several doses (0.01, 0.1, 1, 10, 100, 200 nM) and at three time points (24, 48, 72 h). The results showed that FL118 could effectively inhibit cell viability in both dose- and time-dependent manners (**Figure 1A**). Moreover, 10 nM FL118 showed a similar tumor-cytotoxicity to 100 nM SN-38 at 72 h in CRC cells, indicating that FL118 exerted higher antitumor activity compared to SN-38.

We previously reported that FL118 has an inhibitory effect on non-small cell lung cancer cells [15], we wondered whether FL118 has the same activity on CRC cells. In wound healing assay, HCT-116, SW480 and LOVO cells were treated with FL118 at different concentrations (0, 10, 100 nM) and different time points (24, 48, 72 h), and the results showed that FL118 dramatically suppressed migration of CRC cells (**Figure 1B**). FL118 was demonstrated to inhibit the migration of HCT116 and LOVO cells in both dose- and time-dependent manners.

To explore the effect of FL118 on cell proliferation, BrdU cell proliferation ELISA Kit was employed to examine the proliferation of HCT-116 cells with FL118 treatment at 10 nM for 48 h. The OD value of treated group was lower than normal group ( $P < 0.01$ ) (**Figure 1C**). We found FL118 could obviously inhibit the proliferation of HCT-116 cells.

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**Figure 4.** The effect of CDC73 was tested by blocking experiments. (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ ). CDC73 was blocked by three siRNA. A. qRT-PCR data shows the effect of CDC73 blocked in mRNA level. B. Immunoblotting results illustrated the effect of CDC73 blocked in protein level and its quantified results. C. MTT assay reveals that the OD value was higher than NC group after transferred with siRNA both treated with FL118 at 10 nM. D. BrdU shows the OD value of HCT-116 cells increased after block CDC73 treated with FL118 at 10 nM.

Collectively, these data indicated that FL118 exhibited higher antitumor effect compared with SN-38 in MTT assay and inhibited the cell viability, migration and proliferation in HCT116, LOVO, SW480 cells.

### *The growth of colorectal tumor was inhibited by the treatment of FL118 in vivo*

To evaluate the anti-tumor activity of FL118 on CRC *in vivo*, we conducted human colorectal cancer xenograft in nude mice. As shown in the photographs, the tumor size in FL118-treated group was remarkably smaller compared with control group (Figure 2A). Tumor volume reached 2322.6 mm<sup>3</sup> in control group. However, tumor volume reached 984.61 mm<sup>3</sup> and 537.09 mm<sup>3</sup> in low and high dose group, respectively (Figure 2B). In addition, the weight of nude mice was not influenced by FL118 administration (Figure 2C). Taken together, the result

showed that FL118 exerts a great anti-tumor activity and a low toxicity *in vivo*.

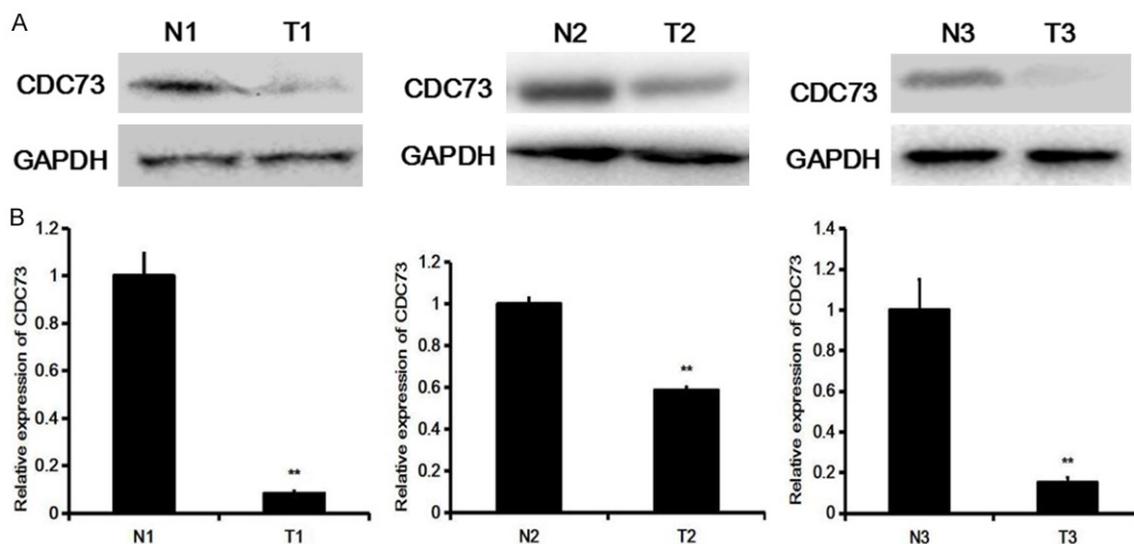
### *FL118 functions as a promoter of CDC73 and an inhibitor of $\beta$ -catenin pathway*

Subsequently, we aimed to explore the detailed molecular mechanism underlying FL118's anti-tumor activity. Mohammad Iqbal Rather has reported that oncogenic microRNA-155 (miR-155) negatively regulates CDC73 expression [16]. Our previous studies have found that FL118 could downregulate oncogenic miR-155 [17], and we wonder to know whether CDC73 is a target for FL118. Therefore, we conducted qRT-PCR and immunoblotting assays to investigate the change in CDC73 expression level *in vivo* and *in vitro*. HCT-116, SW480 and LOVO cells were all treated with FL118 at different concentrations (0, 10, 100 nM) for 48 h. Then, CDC73 level was found to be increased by two folds by FL118 100 nM than control

group at mRNA level in CRC cells ( $P < 0.01$ ) (Figure 3A). Consistently, the CDC73 protein level was also detected to be enhanced by FL118 in a dose-dependent manner (Figure 3B). In addition, HCT-116 xenograft nude mouse experiment showed a similar result to the *in vitro* assay, and the CDC73 level was higher in FL118 groups compared to control group ( $P < 0.01$ ) (Figure 3C and 3D). Richard G has revealed that CDC73 can negatively regulate the  $\beta$ -catenin, which plays an important role in tumor development [14]. In this study, the expression of  $\beta$ -catenin, a key protein in Wnt signaling, was also found to be decreased by FL118 (Figure 3E).

### *The anti-tumor activity of FL118 is mediated by upregulating CDC73 in CRC cells*

In order to investigate whether CDC73 upregulation mediates FL118's antitumor activity, we



**Figure 5.** The expression of CDC73 in colorectal cancer and normal Tissues (N: normal T: tumor). Three patients' tissues were taken from the Affiliated Hospital of Qingdao University. A. Immunoblotting showed the different expression of CDC73 in tumor and normal tissues. B. Corresponding quantitative data are shown as means  $\pm$  SD of three independent experiments (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ ).

conducted a blocking experiment by siRNA in HCT-116 cells. We found that three siRNAs all successfully reduced CDC73 expression both at mRNA (Figure 4A) and protein levels (Figure 4B). One of the CDC73-siRNAs (#600) was selected for subsequent experiments due to its highest inhibitory effect. Next, we conducted MTT and BrdU assays to explore the effect of CDC73 on cell viability and proliferation of HCT-116. MTT results showed that the OD value at 490nm of CDC73-siRNA group was elevated compared to control group ( $P < 0.01$ ) (Figure 4C). Interestingly, the results of BrdU assay were also similar to MTT assay (Figure 4D). The above results indicated that CDC73 plays an indispensable role in FL118 antitumor activity on CRC cells.

#### *CDC73 was down-regulated in human CRC tissues*

To confirm that the expression of CDC73 is differential between cancerous and adjacent tissues, we detected the level of CDC73 in three colorectal carcinoma and their adjacent tissues by western blotting. As shown in Figure 5A, the lower expression of CDC73 was observed in colorectal carcinoma tissues than adjacent tissues. The results of protein quantification also showed a statistical significance in CDC73 protein contents between cancer and normal samples (Figure 5B) ( $P < 0.01$ ).

#### Discussions

FL118, as an inhibitor of survivin, is selected from a high throughput screening of multiple small molecule drug databases. Surprisingly, further study found that FL118 also has a highly selective inhibitory effect against some apoptosis-associated proteins, such as Mcl-1, XIAP and cIAP2, which are closely related to tumor growth and drug resistance [3]. Moreover, it was reported that FL118 can also promote the expression of apoptosis proteins Bax and Bim [15, 18-22], and thus induce cell apoptosis in 293 and IL-3 cells [13, 23, 24]. In addition, the latest research found that FL118 can target oncogenic miR-155 [17]. Apart from the great multitarget effects of FL118, studies have shown that FL118 can overcome irinotecan and topotecan resistance and cisplatin resistance in non-small cell lung cancer cells [15, 25], which are a thorny problem in present cancer clinical therapy, one reason might be because the efflux pump ABCG2 is insensitive to FL118 [19, 20, 25]. Notably, FL118 have been reported to exhibit better antitumor activity than irinotecan and topotecan *in vitro* and *vivo* [19]. However, unlike other camptothecin derivative, FL118 failed to significantly inhibit the activity of TOP1, a well-known camptothecin target [26], which attract us to discover more detailed mechanisms underlying the great antitumor effect of FL118.

In this study, we found that FL118 could significantly inhibit the cell viability, migration and proliferation in CRC cells, and exert better anti-tumor activity compared with SN-38, these results is consistent with previous reports [19]. We also established HCT-116 xenografts model in nude mice, FL118 was confirmed to effectively suppress the tumor growth *in vivo*.

CDC73 is a component of the human Paf1 complex (Paf1C) [27-29] which also consists other four subunits: Ctr9, Leo1, Rtf1, and Paf1 [30, 31], Paf1 complex was reported to be involved in the development of pluripotent stem cells and has multi-functions during transcription [32, 33]. Study have found that CDC73 could interact with RNA polymerase II subunit POLR2A and histone methyltransferase complex, and Set1-like HMTase complex to methylate histone H3 on lysine 4 and induce the activation of transcriptional related activities [21]. CDC73 has been reported to be a tumor suppressor gene in HPT-JT [34, 35] and renal cell carcinoma [36]. Also, over-expression of CDC73 inhibited the growth of HEK293 or NIH3T3 cells [37].

In our work, we found that the expression of CDC73 was significantly lower in CRC tissues compared with their adjacent tissues. Furthermore, FL118 could significantly upregulate the expression of CDC73 both *in vivo* and *in vitro*. With the rise of CDC73, we found FL118 also inhibits  $\beta$ -catenin expression in HCT-116 cells. Next, we employed siRNA interference to reduce the endogenous CDC73 expression, CDC73 knockdown led to the decrease of the efficacy of FL118, suggesting its indispensable role in FL118's antitumor activity.

Recently, many new drugs have emerged in tumor therapy field, and FL118 should gain more attention due to its excellent antitumor properties, including high efficiency, low toxicity and low drug resistance. Our study found that FL118 could significantly inhibit the cell viability, proliferation and migration of CRC cells and tumor growth *in vivo*. Moreover, FL118 may exert its anti-tumor effect by upregulating CDC73, a tumor suppressor and inhibiting Wnt- $\beta$ -catenin pathway. Taken together, our work might provide new insight and evidence on FL118's excellent antitumor mechanism and its further clinical application.

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### Disclosure of conflict of interest

None.

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