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FL118 inhibits viability and induces apoptosis of colorectal cancer cells *via* inactivating the CIP2A/PP2A axis

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ABSTRACT

Aims: FL118, a novel camptothecin analogue, has been extensively studied for its superior antitumor potency. The aim of this research study is to explore its potential mechanism of action in anti- colorectal cancer (CRC).
Main methods: The effect of FL118 on CRC cell proliferation was assessed using CCK-8 assay, while apoptosis was detected using Hoechst staining and Flow cytometry assays. The expression levels of CIP2A were analyzed using qRT-PCR. The expression of CIP2A, PP2A-C, Bax, cleaved caspase-3 and PARP were analyzed using western blotting analysis. The expressions of related proteins in CRC tissues were detected using immunohistochemical staining. TUNEL assay was used to detect apoptosis of tissue. Toxicity of FL118 in primary organs were examined using H&E staining.
Key findings: The results show that FL118 can inhibit the proliferation and clonogenic potential of CRC cells and increase the expression of pro-apoptosis proteins, Bax, cleaved caspase-3 and PARP. Microarray analyses found that FL118 treatment significantly decreases cancerous inhibition of protein phosphatase 2A (CIP2A). Further validation found that CIP2A is aberrantly upregulated in CRC tissues, and is positively correlated with the progression of CRC. *In vitro* findings confirm that FL118 mediates the downregulation of CIP2A, at both protein and mRNA levels. Co-treatment with Okadaic acid (OA) (a PP2A inhibitor) partially abolishes the anti-proliferative and pro-apoptotic effect of FL118. Consistently, *in vivo* experiment demonstrates that FL118 can effectively suppress tumorigenesis without any obvious toxic effects.
Significance: Collectively, these findings exhibit the anti-neoplastic effects of FL118 against CRC through the down regulation of CIP2A, which subsequently enhances the activity of PP2A.

1. Introduction

It has been estimated that 18.1 million newly diagnosed cases and 9.6 million cancer deaths would have been reported worldwide in 2018. Colorectal cancer (CRC) is the third most common type of cancer, and the second leading cause of cancer-related deaths worldwide [1]. In 2018, the number of new cases and deaths from CRC were approximately 1,800,977 (10.0% of total cases) and 861,663 (9% of total cancer deaths), respectively, worldwide [1]. Currently, a comprehensive therapeutic scheme for CRC patients mainly includes surgery, chemotherapy and radiotherapy. Surgical resection is the most effective form of treatment for operable early-stage CRC, and has improved the overall survival of CRC patients. Although profound advances have been made in perioperative management and expansion of screening programs, mortality due to CRC continues to rise globally [2]. It is believed that metastasis and recurrence are crucial events that limit the long-term survival of CRC patients. It has been reported that the incidence of recurrence for stage II–III CRC patients ranges from 29 to 63% [3]. Moreover, overall survival time for patients with liver metastasis is no > 3 years [4]. Hence, screening of novel biomarkers, treatment methods, and therapeutic targets for CRC prevention and treatment are urgently required.

FL118 is a small anti-cancer molecular, which has been identified by Serendipity during high throughput screening of anticancer drugs that

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target the survivin gene. Preclinical studies have reported that FL118 exhibits a superior anti-cancer potency, compared with irinotecan and topotecan [5]. Thus, understanding the molecular mechanisms of the anti-tumor action of FL118 has received considerable attention during recent years. Interestingly, FL118 shares a similar chemical structure with irinotecan and topotecan, which are two FDA-approved anti-cancer drugs for colon cancer treatment. While recent studies have suggested that the mechanisms that underlie the potent antitumor effects of FL118 are distinct from irinotecan and topotecan. While both irinotecan and topotecan are potent inhibitors of topoisomerase 1 (TOP 1) activity, FL118 is a weak inhibitor of TOP 1 [6]. Ling et al. have pointed out that FL118 can selectively inhibit survivin, Mcl-1, XIAP and cIAP2, which may contribute to its anti-cancer effect [7,8]. Similarly, Li et al. also confirmed that FL118 causes tumor regression by targeting multiple distinct mechanisms [8]. FL118 can inhibit tumorigenesis and progression by inhibiting multiple antiapoptotic proteins of the inhibitor of apoptosis (IAP) family and Bcl-2 family, while upregulating pro-apoptotic proteins, Mcl-2 and Bax. p53 is the most frequently detected gene in human cancer, which aids the progression of and causes failure in treatment for patients [9]. Most surprisingly, FL118 selectively targets these genes independently of p53 [8]. Additionally, a study published in 2014 reported that FL118 can promote the senescence of colorectal cancer cells *via* inducing MdmX degradation in a p53-dependent manner [6]. There are still many unanswered questions regarding the molecular mechanism that underpins the anti-cancer effects of FL118 in CRC. Further studies on the current topic are therefore recommended.

Protein phosphatase 2A (PP2A), a serine/threonine phosphatase, is involved in multiple cellular processes. In recent decades, the tumor suppressive role of PP2A has been confirmed in various types of human malignant tumors. It mainly suppresses tumorigenicity by promoting the de-phosphorylation of oncogenes, such as Akt, ERK, c-Myc and p70S6K [10]. Cancerous inhibitor of protein phosphatase 2A (CIP2A), is an endogenous PP2A inhibitor, which inhibits the phosphatase activity of PP2A and subsequently reverses its tumor suppressor function [11]. Numerous studies have demonstrated that the aberrant upregulation of CIP2A is positively correlated with poor prognosis in various human malignancies, including CRC [12–14]. Additionally, Wang et al. reported that temsirolimus can effectively enhance the sensitivity of colon cancer cells towards cetuximab by inhibiting the transcription of CIP2A and promoting its degradation [15].

In the present study, we investigated the effect of FL118 on CRC cell lines and elucidated its underlying mechanisms. *In vitro* results show that FL118 exerts anti-proliferative and pro-apoptotic effects against HCT-116 and SW480 cells. Similarly, FL118 can also effectively suppress tumorigenicity in animal models. Mechanistically, FL118 can indirectly increase PP2A activity by downregulating CIP2A expression in CRC.

2. Materials and methods

2.1. Reagents

FL118, 11-methylenedioxy-camptothecin (Fig. 1A), used in this study was a gift from the American Roswell Park Cancer Institute (RPCI). FL118 was dissolved in dimethyl sulphoxide (DMSO) to obtain the concentration indicated. Antibodies against cleaved caspase-3 (#2302, 1 μg/ml), pro-caspase-3 (#32150, 1:1000), pro-PARP, and cleaved PARP (#32064, 1:1000), CIP2A (#99518, 1:2000), Bax (#32503, 1:1000) and Bcl-2 (#196495, 1:1000), and PP2A (#32104, 1:3000) were purchased from Abcam (Cambridge, UK). Specific antibodies against CIP2A (#14805, 1:1000) and PP2A (#137825, 1:1000) were purchased from Cell Signaling (Danvers, MA, USA), while antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), goat anti-rabbit immunoglobulin horse radish peroxide (IgG-HRP) and anti-mouse IgG-HRP were purchased from Beyotime (Shanghai, China). Hoechst 33258 was purchased from Beyotime (Haimen, China) and PP2A inhibitor OA was obtained from Sigma.

2.2. Clinical samples

This study was approved by the ethics committee of Qingdao University (Qingdao, China) and all participating patients provided written consent. A total of 22 CRC patients who underwent surgical procedures between May 2015 and June 2017 were enrolled in the current study. CRC tissue and adjacent non-cancerous tissue samples were collected and stored at −80 °C until further use. Two independent senior oncologists blinded to the study implemented the diagnosis and staging. Ten out of the 22 tumor samples were pathologically graded as of a low grade (T1 and T2), while the other 12 were graded as being of a high grade (T3 and T4), based on WHO criteria.

2.3. Cell lines and culture

SW480 (#CCL-228) and HCT-116 (#CCL-247) cell lines were acquired from the American Type Culture Collection. McCoy's 5a medium was used to culture HCT-116 cells, while L-15 medium was used to culture SW480 cells. Both mediums were supplemented with 10% fetal bovine serum. All cells were grown at 37 °C in a humidified incubator with 5% CO_2.

2.4. Cell proliferation assay

The effect of FL118 on CRC cell proliferation was assessed using a CCK-8 kit, following the manufacturer's instructions. In brief, 5×10^3 SW480 or HCT-116 cells were plated into 96-well plates. After 24 h of growth, the cells were treated with different concentrations of FL118. After treatment, the cells were washed with 1× PBS, followed by incubation with the CCK-8 reagent. After incubation for 1 h at 37 °C, absorbance was recorded at 450 nm using a spectrophotometer (Texan Group Ltd., Männedorf, Switzerland) to assess proliferation. Cell viability was calculated according to the formula: Cell viability (% of control) = Experimental group (OD value) – Blank group (OD) / control group (OD) – Blank group (OD).

2.5. Hoechst 33258 staining for apoptosis

Trichinella was used to digest the HCT-116 and SW480 cells, then the cells were seeded into a 96-well plate, with 1×10^5 cells in each well. Subsequently, the cells were incubated with Hoechst 33258 (0.5 μg/ml) for 3 min at room temperature. After washing with PBS, the cells were observed and imaged using a microscope.

2.6. Flow cytometry assays

The cells were digested using trypsin, and washed twice in ice-cold PBS. Subsequently, the cells were incubated in Annexin V-FITC and propidium iodide (PI) for 30 mins at room temperature. Then, the rate of apoptosis was testing using flow cytometry (Beckman Coulter Inc., FL, USA).

2.7. Quantitative real-time PCR (qRT-PCR)

A RNeasy Mini Kit (Qiagen 74104) was used to isolate total RNA. 1 μg of RNA from each sample was transcribed into cDNA using a iScript™ cDNA Synthesis Kit (Bio-Rad). Gene expression was analyzed through qRT-PCR using the SYBR Green Master Mix kit (Bio-Rad) on a CFX96 Touch Real-Time PCR Detection System. The expression levels of target genes were normalized to β-actin. The primer sequences used were as follows: CIP2A forward, 5′-CAACCTGGTGTCAGCAGCCTT-3′ and CIP2A reverse, 5′-GCACCGTCCATGTTGACGGTG-3′; Actin forward, 5′- GCTCGTCGTCGACAACGGCT-3′ and actin reverse, 5′-CAAACATGA

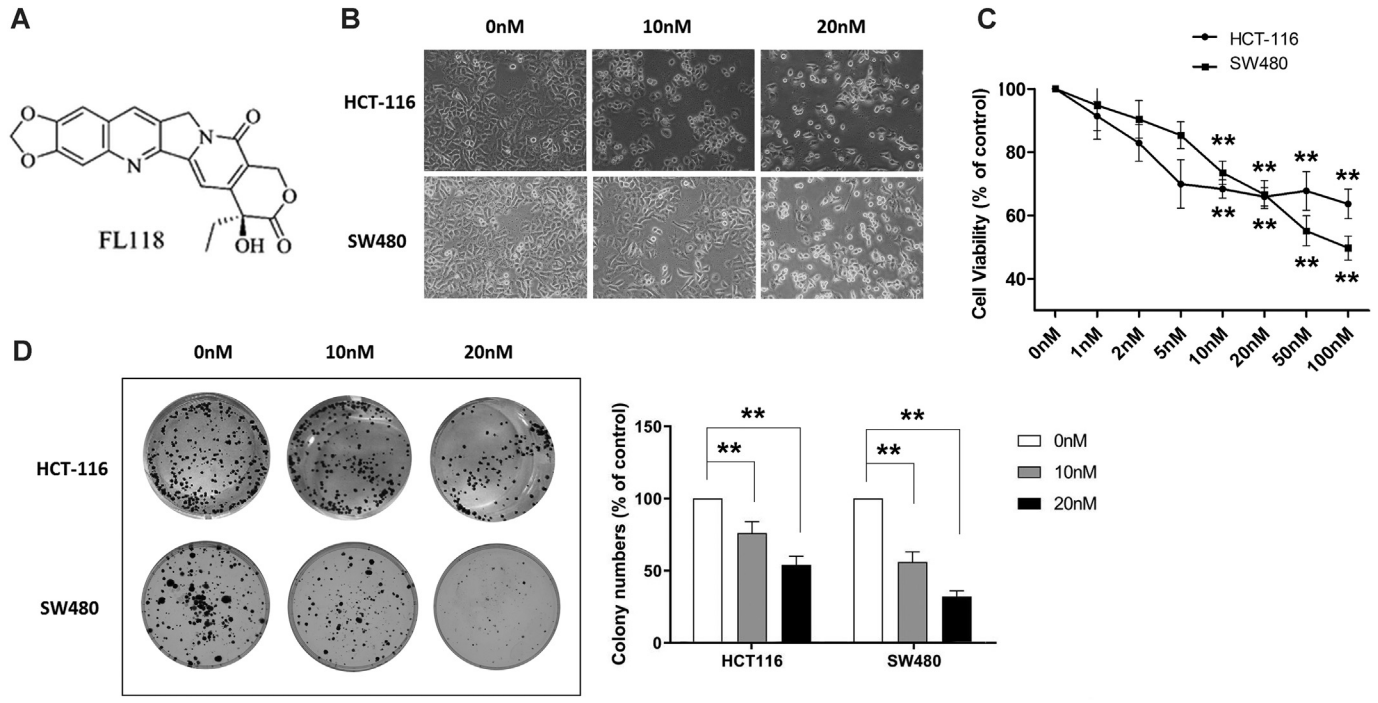

Fig. 1. FL118 inhibits CRC cell growth. A. The chemical structure of FL118. B. The cells indicated were exposed to FL118 (0, 10, and 20 nM) for 48 h. Then, morphological changes of HCT-116 and SW480 cells were observed and photographed under a microscope (Magnification, ×100). C. HCT-116 and SW480 cells were treated with various concentrations of FL118 (0–100 nM) for 48 h. Cell viability was determined using CCK-8 assay. D. Following incubation with FL118 (0, 10, and 20 nM) for 14 days, the numbers of colonies of HCT-116 and SW480 cells were quantified using colony formation assay. ** $P < 0.01$.

TCTGGCTCATCTTCTC-3′.

2.8. Immunohistochemical staining

Immunohistochemical staining of the formalin-fixed, paraffin-embedded section of CRC tissue, adjacent non-cancerous tissue and mice derived tumor tissue was carried out following previously described protocols. In brief, the sections were incubated with antibodies against CIP2A, Ki-67, cleaved caspase-3, Bcl-2 and Bax for 20 min at room temperature. After washing three times with PBS, the sections were incubated with peroxidase-labeled secondary antibodies for 30 mins at room temperature. After three washes with PBS, the sections were incubated with diaminobenzidine, counterstained with Mayer's hematoxylin, dehydrated and mounted. The results of the immunohistochemical staining were independently assessed by two experienced pathologists (Kun-Tu Yeh and Chang Wei-Hsiang), and final scores were agreed upon at a discussion. The staining intensities were scored as $-$, 1^+ and 2^+ for negative, low and high signals, respectively.

2.9. In vivo studies

All animal experiments were approved by the Institutional Animal Care and Use Committee of Qingdao University. In brief, five week old male athymic BALB/c nu/nu mice (18–22 g) were fed sterile food and water in a pathogen-free environment. HCT-116 cells (1×10^7 cells) were injected into the left flanks of the mice. 14 days later, the mice were randomly divided into three groups ($n = 4$) and the following were injected intraperitoneally (i.p.): (i) vehicle group (0.9% sodium chloride plus 1% DMSO); (ii) FL118 low dose group (4 mg/kg/week, dissolved in vehicle, intragastrical administration); and (iii) FL118 high dose group (8 mg/kg/week, dissolved in vehicle, intragastrical administration). Body weight and tumor volume were measured every 4 days until day 42. Cryostat sections (4 μm) of HCT-116 xenograft tumors were stained with H&E to detect morphology, and were measured using TUNEL assay, following protocols described in our preliminary work.

2.10. Statistical analysis

Data are shown as mean ± SD (Standard Deviation) and represent the results from three separate experiments. Student's *t*-test was used to assess differences between two groups. Graph prism (version 7.0) software (La Jolla, CA, USA) and SPSS 20.0 software (IBM, Armonk, NY, USA) were used for statistical analyses. A P value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. FL118 inhibits cell proliferation of CRC cells

FL118 treatment was found to significantly decrease the number, and alter the morphology of cells from both cell lines, compared with the control groups (Fig. 1B). Upon exposure to various concentrations of FL118 (0–100 nM), CCK-8 assay confirmed that FL118 decreases cell viability in a dose-dependent manner ($P < 0.01$) (Fig. 1C). Furthermore, results of the colony formation assay also reveal that FL118 at a concentration of 10 and 20 nM markedly decreases the number of colonies of HCT-116 and SW480 cells (Fig. 1D). Collectively, these results show the anti-proliferative effect of FL118 on CRC cells.

3.2. FL118 promotes the apoptosis of CRC cells

Next, we determined whether apoptosis contributes to the inhibitory effect caused by FL118 on the growth of HCT-116 and SW480 cells. Hoechst 33258 staining results indicate that fragmented nuclei and a condensed chromatin pattern significantly increase in FL118-treated HCT-116 and SW480 cells (Fig. 2A). Flow cytometry confirmed that FL118 is responsible for the apoptosis of HCT-116 and SW480 cells in a dose dependent manner. In HCT-116 cells, FL118 (0, 10, and

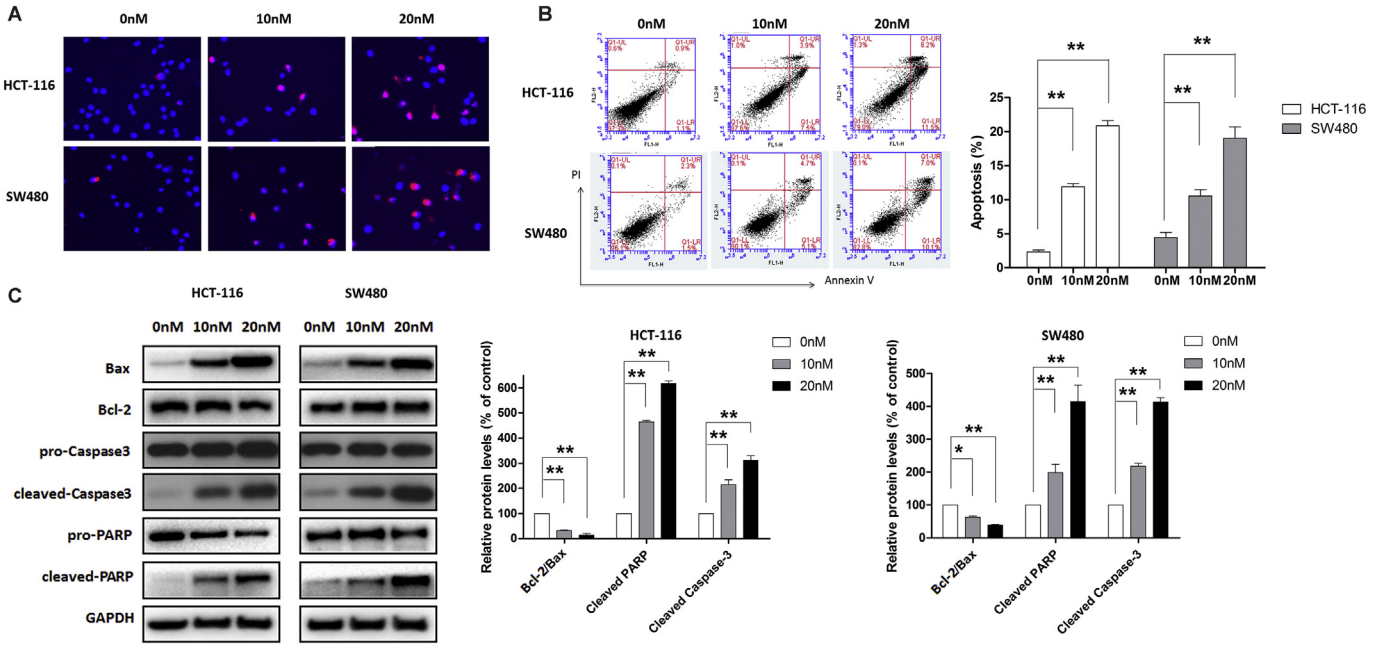

Fig. 2. FL118 promotes apoptosis of CRC cell lines. HCT-116 and SW480 cells were treated with FL118 (0, 10 and 20 nM) for 48 h. A. Apoptosis analysis of FL118 treated cells using microscopy with Hoechst 33258 staining (magnification, 200×). Hoechst 33258 staining enrichment shows the apoptotic cells. B. Following Annexin-FITC/PI staining, the apoptotic cell ratio was quantified using flow cytometry. C. Western blotting analysis of the expression level of apoptosis-related genes (Bax, Bcl-2, pro-caspase-3, cleaved caspase-3, pro-PARP and cleaved PARP) in the cells indicated. GAPDH was chosen as the internal control. $^{**}P < 0.01$.

20 nM) induced apoptosis rates were 2.0%, 11.4% and 22.3%, respectively, while in SW480 cells the rates were 3.8%, 9.8%, and 17.1%, respectively ($P < 0.01$) (Fig. 2B). We also examined expression levels of several apoptosis-related genes, including Bax, Bcl-2, pro-caspase-3, cleaved caspase-3, pro-PARP and cleaved PARP. As expected, FL118 treatment markedly increases casapse-3 activation and PARP cleavage, while decreasing the Bcl-2/Bax ratios ($P < 0.01$, Fig. 2C). Taken together, the above data show the pro-apoptotic property of FL118 in CRC cells.

3.3. FL118 inactivates the CIP2A/PP2A signaling pathway in CRC cell lines

In order to further elucidate the mechanism underlying the anti-proliferative and pro-apoptotic effects of FL118, a microarray analysis was carried out to identify differentially expressed proteins in cells treated with FL118. As shown in Fig. 3A, 131 proteins with a fold change of 1.5 and $P < 0.01$ were identified as differentially expressed proteins in FL118 treated-cells, compared with the control. CIP2A is one of the most significantly down-regulated genes. Given its oncogenic role in various malignant tumors, we chose to perform the subsequent experiments using CIP2A as a potential target. As expected, further validation demonstrates that FL118 significantly decreases the mRNA expression of CIP2A in both HCT-116 and SW480 cells, in a dose-dependent manner ($P < 0.01$, Fig. 3B). Similarly, western blotting analysis also shows the decrease in CIP2A protein expression following FL118 treatment ($P < 0.01$, Fig. 3C). Accumulating evidence has confirmed that CIP2A promotes tumorigenesis and progression by inhibiting the activity of PP2A-C [16–18]. Therefore, we also examined whether FL118 affects the expression level and activity of PP2A-C in HCT-116 and SW480 cells. Interestingly, our data show that FL118 significantly increases PP2A-C activity ($P < 0.01$), without altering its protein expression ($P > 0.05$) (Fig. 3C and D). Overall, these results suggest that FL118 treatment can enhance PP2A-C activity by inhibiting the expression of its endogenous inhibitor, CIP2A.

3.4. CIP2A is aberrantly up-regulated in CRC

In order to elucidate the expression of CIP2A in CRC tissues, we analyzed its expression in 163 CRC tissues and 10 para-carcinoma tissues of The Cancer Genome Atlas (TCGA) database. The analysis revealed significant increase in CIP2A expression in CRC tissues, in comparison with that of adjacent normal tissues ($P < 0.001$, Fig. 4A). Consistent with the results from TCGA database, we found that at both mRNA ($P < 0.01$, Fig. 4B) and protein level, CIP2A expression is higher in CRC tissues than in corresponding non-cancerous tissues ($P < 0.01$, Fig. 4C and F). In addition, CIP2A expression is positively correlated with tumor stage (stage I and stage II) ($P < 0.01$, Fig. 4D) and lymph node metastasis ($P < 0.01$, Fig. 4E) in CRC patients. CIP2A expression is higher in patients with high grade CRC (stage III and stage IV) than low grades (stage I and stage II).

3.5. Inhibition of PP2A activity partially reverses the effect of FL118 on CRC cell lines

To substantiate these results that the increased activity of PP2A was contributed the tumor suppressive role of FL118 in CRC cells. Okadaic acid (OA) and LB-100, two commonly used PP2A inhibitors, were used to block the activity of PP2A in HCT-118 and SW480 cells. As illustrated in Fig. 5A, no obvious change was detected in the expression of CIP2A and PP2A-C, between the control and OA treated groups ($P > 0.05$, Fig. 5A). Meanwhile, treatment with OA partially reversed the decrease in CIP2A expression by FL118 ($P < 0.01$, Fig. 5A). The activity of PP2A-C in the OA group was lower than in the control group ($P < 0.01$, Fig. 5B), indicating effective PP2A-C inhibition. When HCT-116 cells and SW480 cells were co-treated with FL118 and OA, increased activity of PP2A induced by FL118 treatment was at least partially abolished ($P < 0.01$, Fig. 5B). Furthermore, OA exposure effectively alleviated the growth inhibition effect of FL118 ($P < 0.01$, Fig. 5C and Fig. S1A). Similarly, inhibition of PP2A activity by OA abrogated FL118-induced apoptotic cell death (Fig. 5D and Fig. S1B). The apoptosis rates of HCT-116 and SW480 cells in the FL118 and FL118 + OA groups were 22% and 12.2%, and 18.2% and 8.0%,

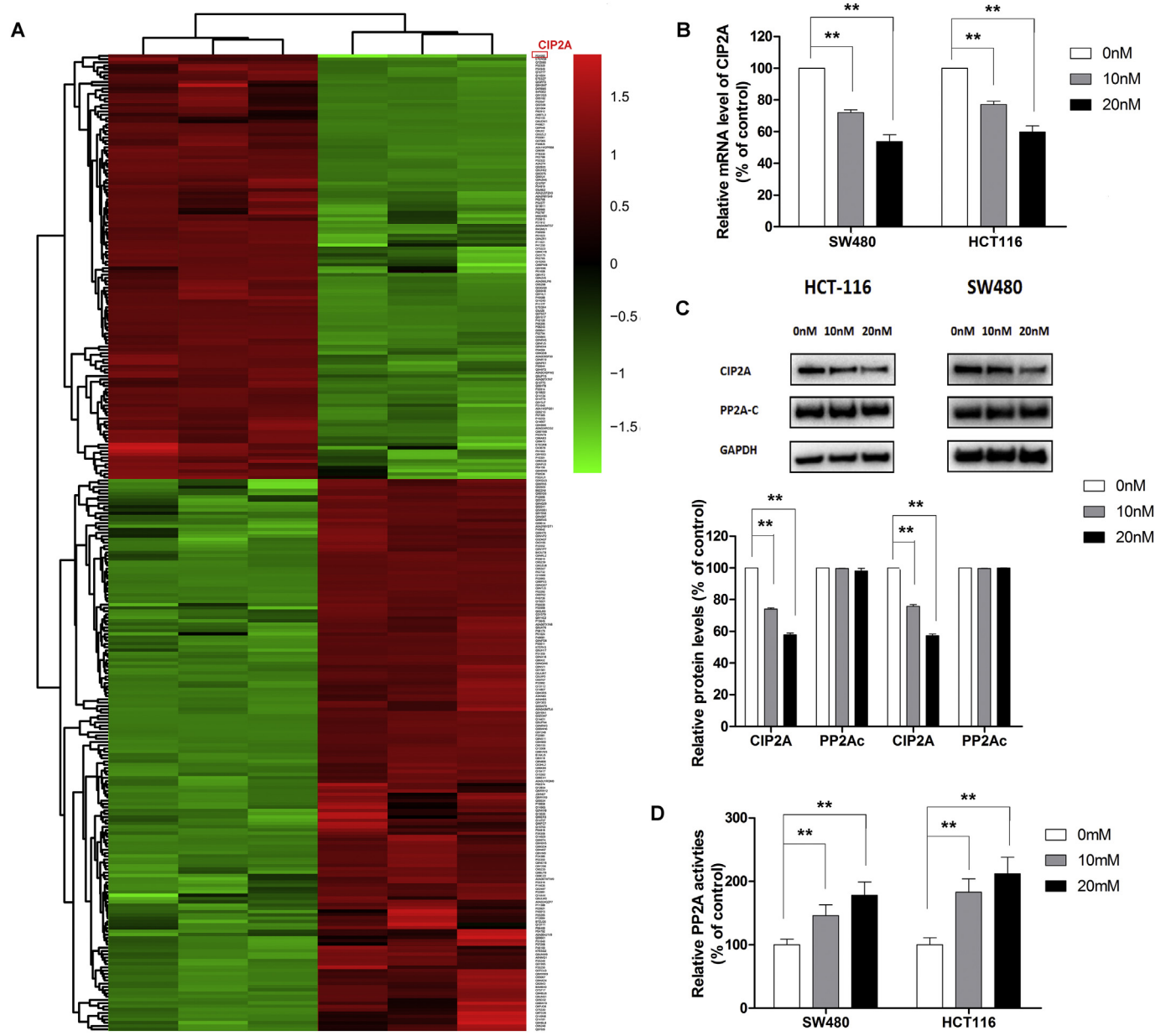

Fig. 3. FL118 treatment reduces the expression level of CIP2A in CRC cell lines. A. The heat map of the microarray analysis shows the differentially expressed genes in HCT-116 cells. CIP2A is one of the downregulated genes in FL118-treated cells. B. Upon exposure to FL118 (0, 10 and 20 nM) for 48 h, the relative expression mRNA level of CIP2A in HCT-116 and SW480 cells was determined using qRT-PCR. C. Western blotting analyzes of the expression level of CIP2A and PP2A-C in the cells indicated. GAPDH was chosen as the internal control. D. The relative expression of PP2A phosphatase activity in FL118-treated cells. $^{**}P < 0.01$.

respectively ($P < 0.01$, Fig. 5D). Co-treatment with OA could partially reverse the decrease in Bcl-2/Bax ratio and increase cleaved caspase-3 and cleaved PARP levels induced by FL118 ($P < 0.01$, Fig. 5E). Furthermore, a PP2A-c specific targeted siRNA was employed to knockdown its level in CRC cells ($P < 0.01$, Fig. S1C). Consistently, knockdown of PP2A-c also could obviously alleviate the anti-proliferative and pro-apoptotic effects of FL118 ($P < 0.01$, Fig. S1C and Fig. 2D). Collectively, these findings indicate that the increased activity of PP2A contributes to the tumor suppressive role of FL-118 in CRC, at least partially.

3.6. FL118 suppresses tumorigenesis in the CRC xenograft model

A CRC xenograft model was established to examine the anti-neoplastic effect of FL118 *in vivo*. When the tumor volume reached 100 mm^3, tumor bearing mice were divided into the following three groups: vehicle group, low dose group (4 mg/kg/week) and high dose group (8 mg/kg/week). The tumor volume and body weight of the mice were measured every four days. As shown in Fig. 6A and B, at both a low dose and a high dose, FL118 can effectively delay tumor growth, compared with the vehicle control group ($P < 0.01$, Fig. 6A and B). TUNEL assay and IHC results clearly demonstrate that FL118 triggers cell apoptosis and inhibits the growth of tumors in mice. A significant increase in TUNEL-positive cells, cleaved caspase-3, Bax, and decrease in ki-67 and anti-apoptotic Bcl-2 (Fig. 6C and D) were found. Consistent with the *in vitro* findings, expression of CIP2A in mouse tumor tissues was found to have dramatically decreased in the FL118 treatment group, in comparison with the control group (Fig. 6D). As shown in Fig. 6E, no significant change in body weight was detected in mice treated with FL118. H&E staining revealed that FL118 did not cause any pathological changes in the heart, liver, spleen, lung and kidneys (Fig. 6F). Taken together, these results show that FL118 effectively

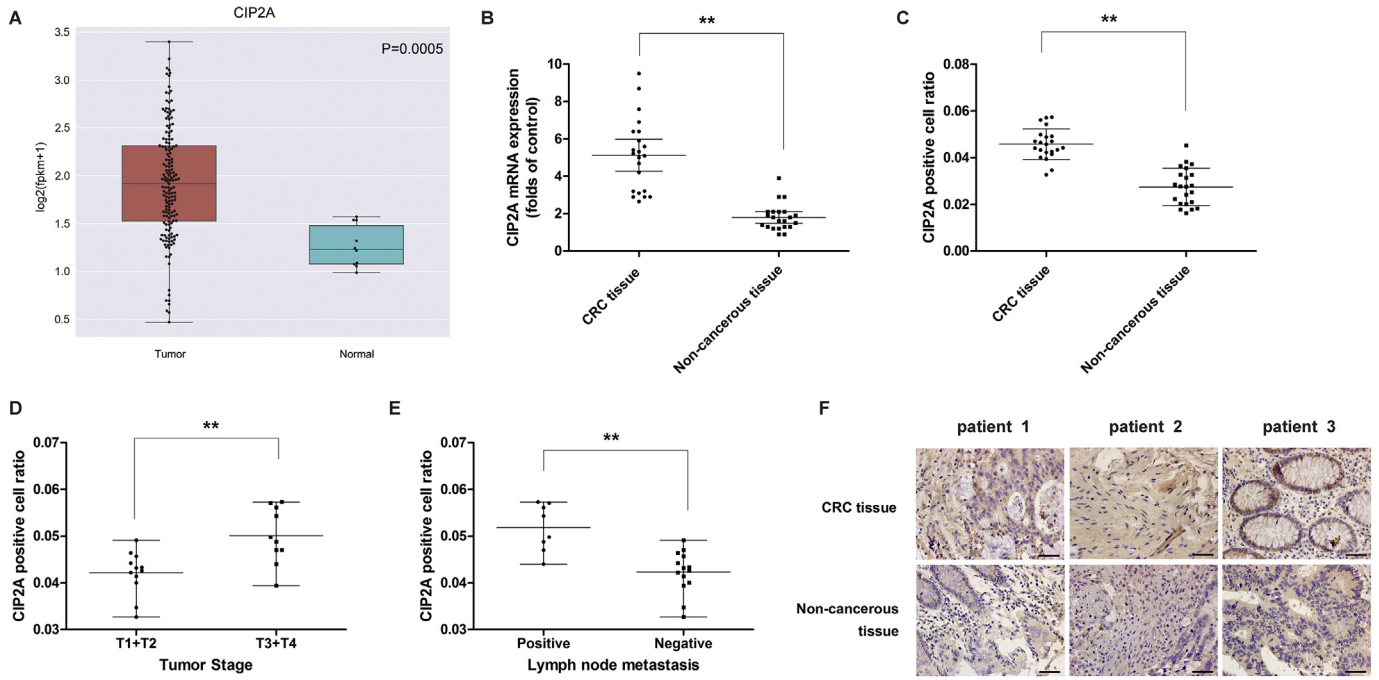

Fig. 4. CIP2A is up-regulated in CRC tissues and is correlated with poor prognosis. A. CIP2A expression profiles of 163 CRC and 10 adjacent-normal tissues of The Cancer Genome Atlas (TCGA) database which were analyzed. B. Validation of the relative mRNA expression level of CIP2A in 22 CRC tissues and adjacent non-cancerous tissues were measured using qRT-PCR. C. The CIP2A positive ratio reflects the abundance of CIP2A in 22 CRC tissues and adjacent non-cancerous tissues, as analyzed using immunohistochemistry. D. High CIP2A levels are positively correlated with tumor stage. CIP2A expression level in high grade (T3 and T4) CRC tissue is higher than that of low grade (T1 and stage T2) CRC tissue. E. Lymph node metastasis is positively correlated with lymph node metastasis. F. Representative images of CIP2A staining of the tissues of 3 CRC patients. $^{**}P < 0.01$.

suppresses tumorigenesis *in vivo* without causing toxicity, indicating that FL118 is a potentially safe and promising anti-tumor agent.

4. Discussion

The tumor suppressive role of FL118 has been studied extensively during recent times in many types of cancers, owing to its superior antitumor potency and good tolerance. It has been reported that FL118 suppresses cell growth and metastasis *via* elevating the level of GYGB. Recent studies on the anti-tumor effects of FL118 have mainly focused on head-and-neck cancer, and rarely on, CRC. Despite being an analogue of irinotecan and topotecan, it is now accepted that the mechanisms that underlie the potent anticancer effect of FL118 is distinct from that of these two anti-cancer agents. In the present study, the results demonstrate that FL118 effectively inhibits proliferation and induces apoptosis in CRC cell lines. CIP2A was found to be aberrantly increased in CRC tissues and cells and was positively correlated with poor prognosis in CRC patients. Additionally, FL118 was able to significantly decrease CIP2A levels and increase PP2A activity, which contributed to its anti-cancer properties.

Apoptotic cell death is an effective strategy for killing tumor cells [19]. It is mainly regulated by the balance between pro-apoptosis and anti-apoptosis proteins. An early study by Rabi et al. reported of the pro-apoptotic role of FL118 in pancreatic cancer cells which proceeds through the down-regulation of anti-apoptotic survivin and XIAP, and up-regulation of Bax [20]. Consistent with published findings, the findings of this study also validate the apoptosis-inducing effect of FL118, shown by the increase in Bax, cleaved caspase-3 and cleaved PARP, as well as decrease in Bcl-2 levels. In addition to Bax and Bcl-2, multiple anti-apoptotic proteins (survivin, XIAP, cIAP2 and Mcl-1) and pro-apoptotic proteins (Bim) are also regulated by FL118 [8], which suggests that FL118 can regulate apoptosis by modulating multiple targets.

It has been well-documented that the aberrant overexpression of CIP2A can promote cell proliferation and metastasis by inhibiting PP2A activity, which leads to the phosphorylation of several oncogenes, including c-Myc, Akt, Plk1, E2F1 and mTORC1 [11]. A large body of literature has reported that knockdown of CIP2A expression can effectively suppress tumor growth, metastasis, as well as increase the sensitivity of CRC cells to 5-fluorouracil and oxaliplatin [21]. Hence, CIP2A is a safe and promising therapeutic target for CRC. In the current study, it was demonstrated that CIP2A levels are dramatically reduced in FL118-treated cells at both mRNA and protein levels. Consistent with previous findings, the level of CIP2A was found to be significantly higher in CRC tissues, than in adjacent non-cancerous tissues. Furthermore, patients with higher CIP2A expression manifested a high tumor stage, with lymph node metastasis. Interestingly, the results also reveal that FL-118 treatment markedly enhances the activity of PP2A, while no significant change in the protein expression of PP2A was detected. Thus, we concluded that the CIP2A/PP2A axis is, at least, partially responsible for the anti-cancer effect of FL118 in CRC. This notion is further supported by the reversal of the effects of FL118 observed through the pharmacological inhibition of PP2A by OA. Multiple chemotherapeutic agents, such as checkpoint kinase inhibitors [22], afatinib [23] have been reported to kill tumor cells or enhance the sensitivity of other agents by downregulating CIP2A and increasing PP2A activity. Similarly, various compounds, including Arctigenin and cucurbbitacin B [24,25], have also been found to exert a tumor suppressive role by targeting CIP2A, which subsequently reactivates PP2A. Nevertheless, little is known of how FL118 downregulates CIP2A levels, as well as the downstream signaling pathway of the CIP2A/PP2A axis in CRC. A recent study reported that temsirolimus can decrease CIP2A levels *via* inhibiting its transcriptional activity and enhancing degradation through the lysosomal-autophagy pathway in CRC [15].

5. Conclusion

In the present study, our results show that FL118 can effectively

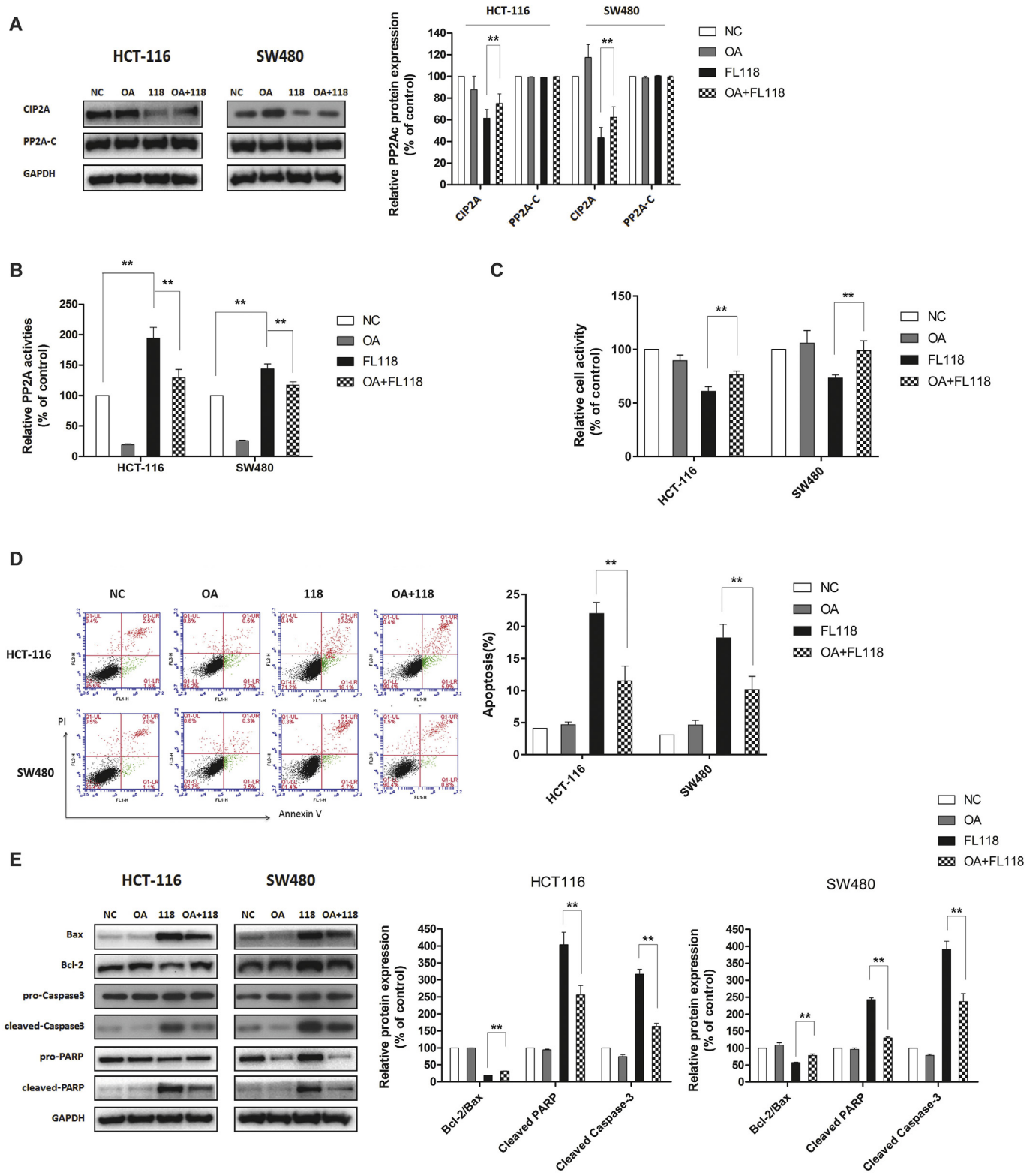

Fig. 5. Inhibition of PP2A activity abrogates the anti-proliferative and pro-apoptotic effects of FL118 in CRC cell lines. HCT-116 and SW480 cells were pretreated with or without the PP2A inhibitor (OA), followed by incubation with FL118 (20 nM) for 48 h. A. Western blotting analyzes of the relative expression levels of CIP2A and PP2A. GAPDH was used to normalize the level of protein. B. Phosphatase activity of PP2A in the cells indicated was detected using an ELISA kit. C. Cell viability was evaluated using CCK-8 assay. D. Analysis of apoptotic cell ratio using flow cytometry, following Annexin-FITC/PI staining. E. Western blotting analyzes of the expression level of apoptosis-related genes (Bax, Bcl-2, pro-caspase-3, cleaved caspase-3, pro-PARP and cleaved PARP) in the cells indicated. GAPDH was chosen as the internal control. $^{**}P < 0.01$.

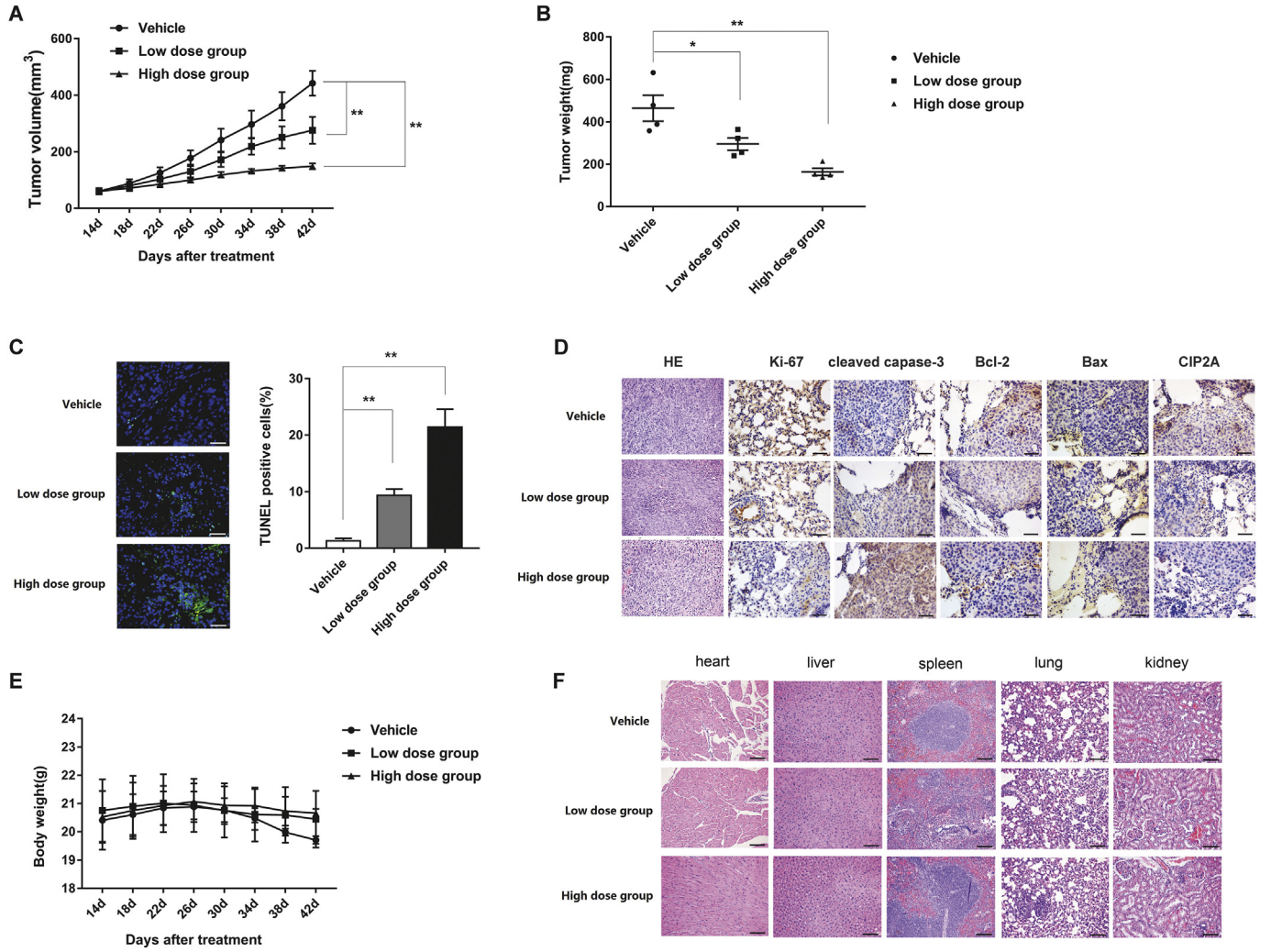

Fig. 6. FL118 suppresses tumor growth *in vivo*. BALB/c nude mice were subcutaneously injected with HCT-116 cells. Then, the HCT-116 cell tumor bearing mice were treated with vehicle, low dose FL118 or high dose FL118 for 4 weeks. A. Tumor volume was calculated every 4 days. B. Tumor weight was recorded on the 42nd day. C. Apoptotic cells in tumor tissues were identified using TUNEL staining. D. The expression level of Ki-67, cleaved caspase-3, Bcl-2, Bax and CIP2A in tumor tissues were analyzed using IHC staining. H&E staining was used to observe the histological characteristics of tumor specimen sections. E. The body weight of the tumor bearing mice was recorded every 4 days. F. The toxicity of FL118 on the heart, liver, spleen, lung and kidneys were examined using H&E staining. $^{*}P < 0.05$; $^{**}P < 0.01$.

suppress cell growth and promote apoptosis in CRC cells. Consistently, *in vivo* studies also confirmed that FL118 causes obvious tumor regression in a xenograft model. The results also identified that FL118 exhibits an anti-tumor effect against CRC *via* targeting CIP2A, in order to enhance the activity of PP2A. Collectively, current findings provide new insights into the role of FL118 as a superior anticancer drug and support its further development for CRC treatment in clinical practice. Future studies are required to better understand the mechanism by which FL118 modulates the CIP2A/PP2A axis.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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