

## Original Article

# Preclinical activity of the rational combination of apatinib in combination with CPT-11 in KRAS-mutant colorectal cancer patient-derived xenograft model

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**Abstract:** Colorectal cancer (CRC) is a leading cause of cancer deaths. Despite the introduction of targeted therapies in clinical practice for CRC, the overall outcome of CRC is not satisfactory, owing to tumor recurrence and distant metastasis. Treatment of metastasis CRC patients with KRAS mutation with anti-angiogenic drugs, has been shown to bring significant advances and represents standard of care. However, drug resistance ultimately develops and new treatment strategies are urgently required. The Patient-derived xenograft (PDX) model is an important drug evaluation animal model research tool for more personalized precision medicine. In this study, one KRAS-mutant CRC PDX model was established. We investigated antitumor response of a novel selective VEGFR2 inhibitor apatinib alone or in combination with CPT-11 in the CRC PDX model. Our study using PDX model of KRAS-mutant CRC showed that combination treatment significantly inhibited the growth of tumors, reduced micro vessel density, suppressed proliferation, and increased apoptosis. Taken together, these preclinical data may suggest a rational combination strategy with apatinib and CPT-11 for patients with KRAS mutant CRC.

**Keywords:** KRAS-mutant, apatinib, CPT-11, colorectal cancer, PDX

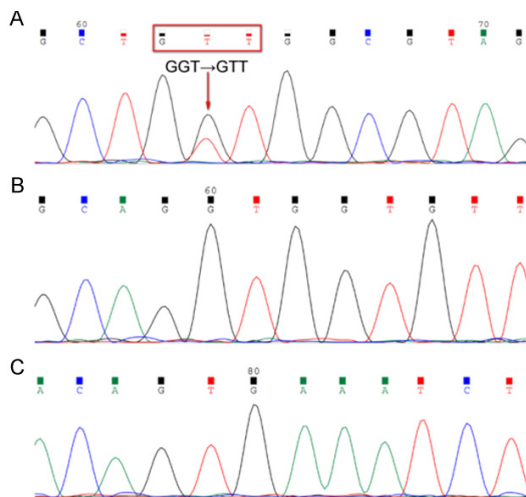
## Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers, with over one million new cases each year worldwide [1]. In China, with the changed lifestyle, there is an increasing trend in its incidence and mortality rate during the past years [2]. Despite the encouraging advances in widespread screening and therapeutic techniques have been achieved during the past two decades [3-5], Approximately 25% of CRC patients are found in distant metastasis stage at initial diagnosis and almost 50% of CRC patients will develop metastases, the prognosis of those with advanced or metastatic disease remains poor [6-8].

Angiogenesis is a well-recognized hallmark of cancer [9], which is involved in tumor invasion, metastases and risk for recurrence [10-13]. Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are known to play an

important role in angiogenic processes through both direct and indirect mechanisms [14-16]. Treatment of patients with metastatic CRC patients with anti-angiogenetic agents has been shown to induce significant clinical benefit and represents standard of care. VEGF inhibitors, including bevacizumab, aflibercept, regorafenib and recently ramucirumab, have significantly improved both overall survival (OS) and progression-free survival (PFS) in different lines of treatment for patients with metastatic CRC [16-20]. Despite of these advances, tumors ultimately become resistant to these agents. Therefore, the development of novel anti-angiogenic agents is urgent needed [21-23].

Apatinib, also known as YN968D1, is a novel and highly selective inhibitor of VEGFR2 tyrosine kinase. Previous studies indicated that Apatinib had an antitumor effect in a wide variety of solid tumors in vitro and in vivo [24, 25]. It was approved for clinical use in China as a



**Figure 1.** Sequencing analysis of KRAS, NRAS and BRAF genes. A. Sequencing analysis of KRAS exon 2. Missense mutation (GGT→GTT) was observed. B. Sequencing analysis of NRAS exon 2. No mutations were found. C. Sequencing analysis of BRAF exon 15. No mutations were found.

third-line or later treatment for patients with metastatic gastric cancer in 2014 [26]. In addition, it is currently being evaluated in Phase II/III clinical trials for multiple cancers including lung cancer and breast cancer [27, 28]. For its positive clinical value and subliminal demand, apatinib as a promising anti-angiogenesis inhibitor deserved further development.

CPT-11 is an intravenous camptothecin analog, as a single agent and in combination with chemotherapies, has been widely used to treatment for advanced colorectal [29, 30] and gastric cancer [31]. In the present study, we evaluated the antitumor effects of a small molecule inhibitor apatinib in combination with CPT-11 in the patient-derived xenograft model of colorectal cancer.

## Materials and methods

### Drugs

Apatinib was obtained from Jiangsu Hengrui Medicine, Co. (Lianyungang, China). CPT-11 was purchased from Pfizer, Inc. (NY, USA). Apatinib was diluted in 0.5% (w/v) carboxymethyl cellulose, and CPT-11 was dissolved in physiological saline.

### Patient and tumor tissues

Tumor specimens were obtained at initial surgery from a 38-year-old male colon carcinoma

patient. Informed consent was signed by the patient and the study received ethics board approval at First Affiliated Hospital, College of Medicine, Zhejiang University. The histological type was determined according to WHO criteria. The tumor was diagnosed as middle differentiated adenocarcinoma (T4N1M1).

### Genomic DNA preparation and mutation analysis

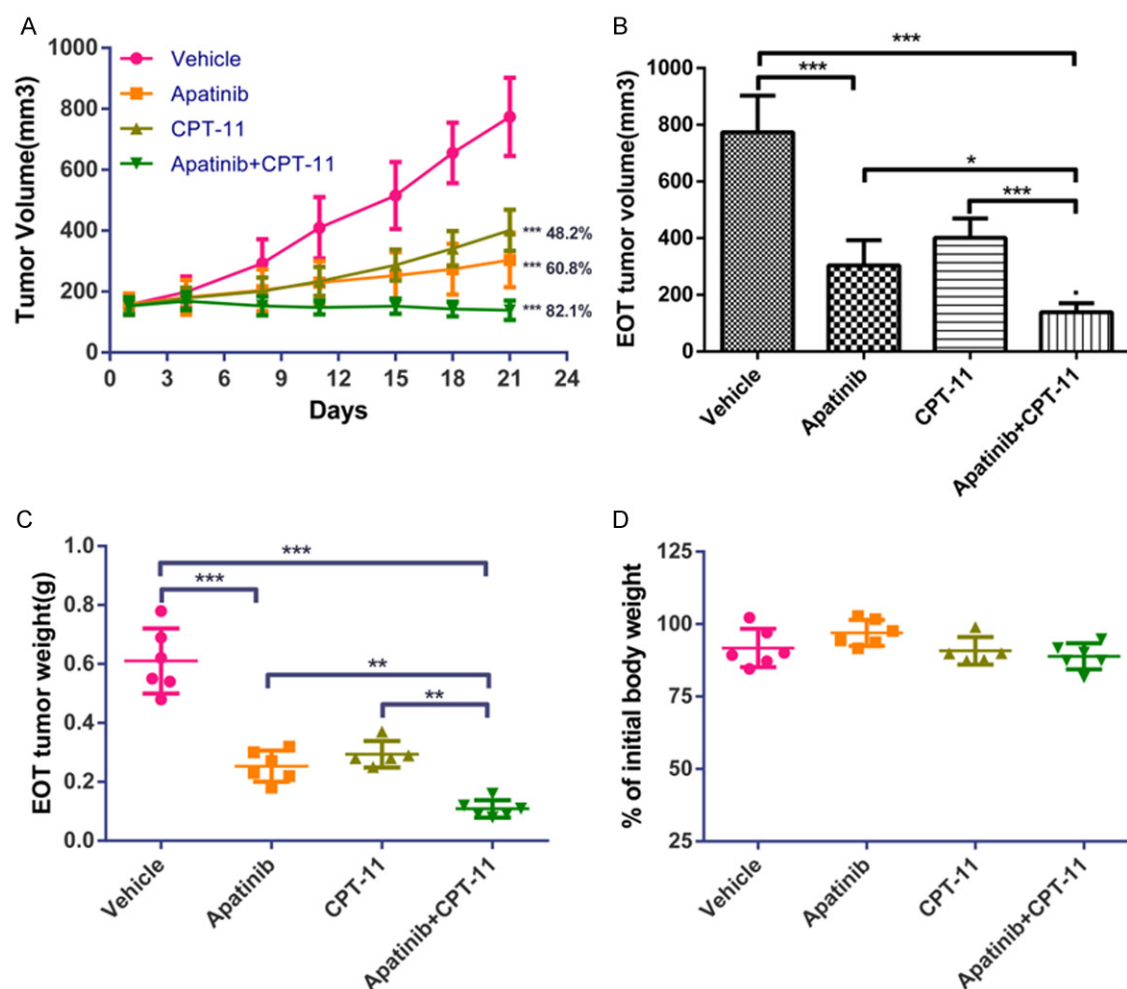
Tumor samples were harvested from euthanized mice, and pathologically reviewed to ensure that no significant tumor necrosis had occurred before extraction of DNA. Genomic DNA samples were isolated by DNeasy Blood & Tissue Kit (Qiagen, CA, USA) according to the manufacturer's instructions. Polymerase chain reactions (PCR) of KRAS exon2, NRAS exon2, and BRAF exon15 were performed as previously described [32]. PCR products were sequenced at TSINGKE Biological Technology (Beijing, China).

### Establishment of PDX model

Five-to-six-week-old female BALB/c nude mice were purchased from Shanghai Slac Laboratory Animal Corporation (Shanghai, China), were housed in a temperature-controlled animal facility on a 12 of 12 hours light/dark schedule with food and water ad libitum. PDXs were established using fresh CRC tissues surgically removed from a patient with CRC as previously described [33]. The use of experimental animals adhered to the Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985). All animal studies were approved by the Institutional Animal Care and Use Committee of Zhejiang University (approval ID: SYXK (ZHE) 2005-0072).

### Treatment protocol

Xenografts from the third generation were used for the drug experiments, once the tumor volume had reached about 100-200 mm<sup>3</sup>. Mice were randomized into four groups (5-6 mice per group): (A) vehicles administrated orally gavage and i.v.; (B) apatinib, 150 mg/kg, oral gavage, daily; (C) CPT-11, 30 mg/kg, i.v., twice per week; (D) apatinib + CPT-11. Mice were treated during 21 days. Mice were monitored regularly for their body weight and tumor growth with a Vernier caliper twice every week. Tumor volume (mm<sup>3</sup>) was calculated by the following formula:  $a \times b^2/2$ , where a and b refer to the larger and



**Figure 2.** Antitumor activity of apatinib alone or in combination with CPT-11 in the PDX model of CRC. Mice (5-6 mice/group) were treated with vehicle, apatinib, CPT-11 or apatinib and CPT-11. A. Tumor growth curve of PDX model. B. Endpoint tumor volume (mm<sup>3</sup>) of PDX model. C. Endpoint tumor weight (g) of PDX model. D. Mice body weight represented as the percent initial body weight at day 21 compared to day 1. There were no statistically significant differences in body weight at the end of each treatment group. Bars, mean  $\pm$  SD. \* $P$  < 0.05; \*\* $P$  < 0.01; \*\*\* $P$  < 0.001. EOT, end of treatment.

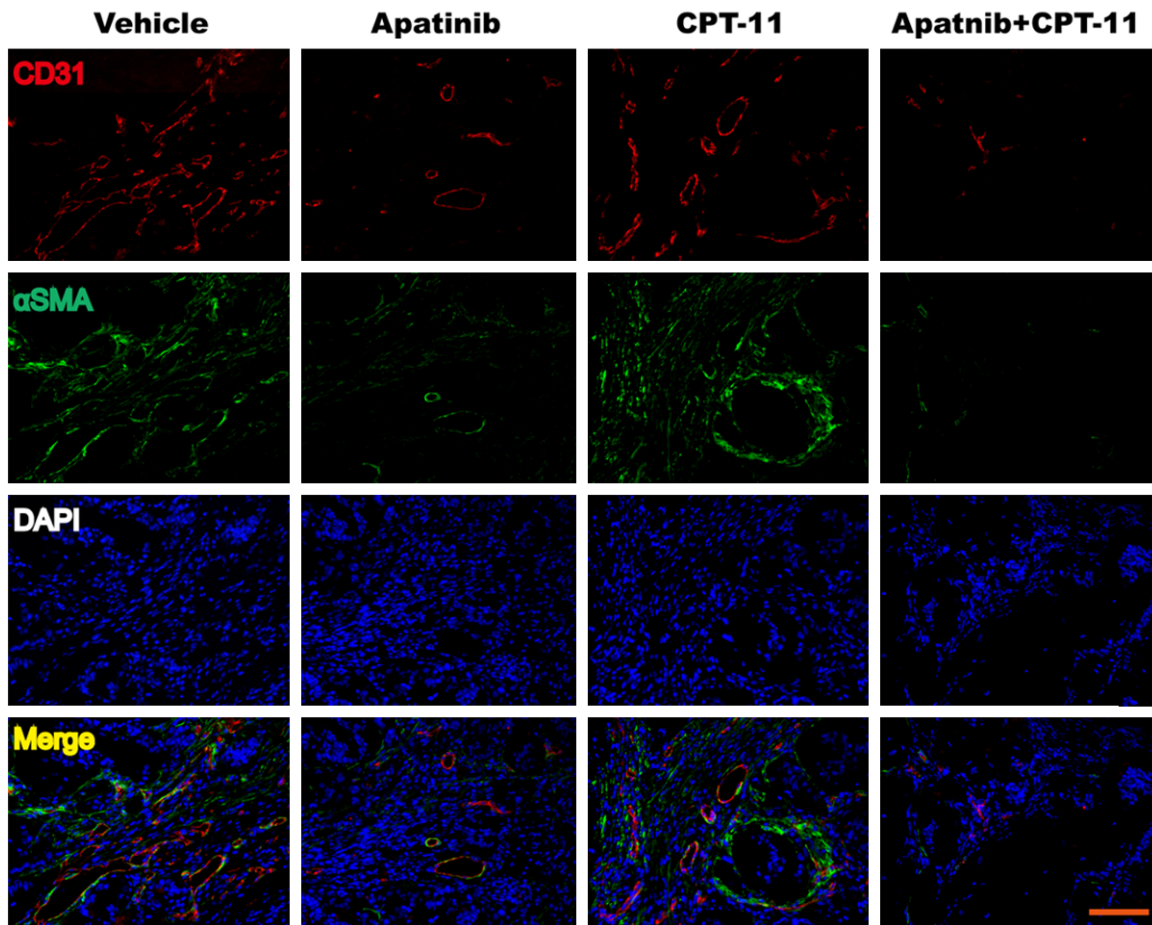
smaller dimensions respectively collected at each measurement. Relative tumor growth inhibition (TGI) was calculated using the following formula:  $(1-T/C)\%$ , where T is relative tumor growth of treated group, and C is relative tumor growth of control group. The mice were sacrificed after 21 days of treatment. Tumors were excised, weighed, and either processed for paraffin embedding or snap-frozen and stored in liquid nitrogen and used for further experiments.

#### Immunohistochemistry (IHC)

Xenograft specimens were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, dehydrated and embedded in paraffin (for 4  $\mu$ M serial

sections). Immunostainings were performed using the following primary antibodies: VEGF (Abcam, Cambridge, United Kingdom), VEGFR2 (Abcam, Cambridge, United Kingdom), MMP9 (Abcam, Cambridge, United Kingdom) and Ki-67 (Abcam, Cambridge, United Kingdom). Staining procedures were conducted according to manufacturer's protocol. The slides were photographed using an Olympus BX60 (Olympus, Japan).

The intensity of VEGF, VEGFR2 and MMP9 was evaluated as previously reported [34-36]. For Ki67, only nuclear immunoreactivity was considered positive. The percentage of Ki67-stained nuclei was determined by counting at least five randomly chosen microscopic fields



**Figure 3.** Apatinib in combination with CPT-11 significantly inhibited tumor angiogenesis in the PDX model of CRC. Representative pictures of blood vessels are shown. Vasculature was examined with immunostainings for endothelial cells (anti-CD31 antibody, green) and pericytes (anti- $\alpha$  SMA antibody, blue). DAPI counterstain marks nuclei in blue. Magnification:  $\times 200$ ; scale bar, 100  $\mu$ m.

(magnification 40  $\times$ ) and 500 total cells. All samples were analyzed and evaluated independently by two board-certified clinical pathologists blinded to the clinical and molecular characteristics of the patient.

#### Fluorescent immunohistochemistry

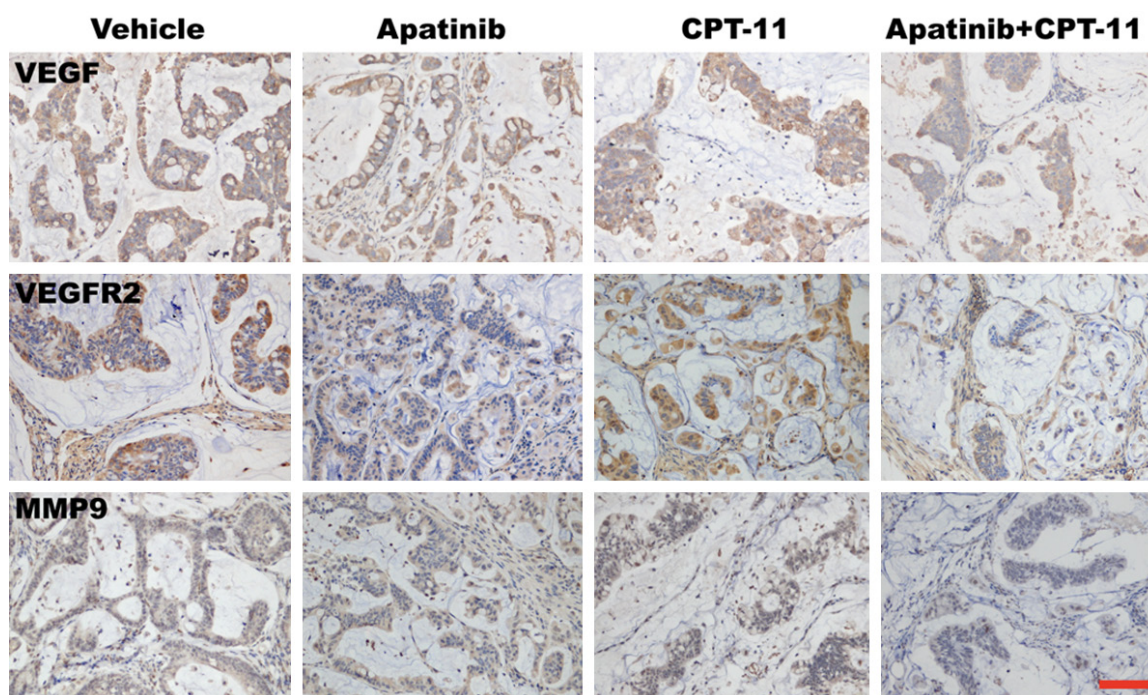
Tumor-bearing mice were anesthetized and the tumors harvested. Tumor specimens were then fixed in 4% PFA overnight at 4°C, rinsed several times with PBS, infiltrated with 30% sucrose, frozen in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Inc, Torrance, CA) and frozen for cryostat sectioning. For immunofluorescence staining, 5- $\mu$ m thick sections of tumor samples were incubated with anti-CD31 (Biolegend, San Diego, CA) and anti-SMA (Abcam, Cambridge, United Kingdom) as the primary antibodies. After washing, sections were incubated with fluorescent (Cy3- or FITC-

conjugated) secondary antibodies (goat anti-rat or goat anti-rabbit). Fluorescent secondary antibodies were purchased from Jackson Immuno Research (West Grove, PA). Sections were mounted using Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired under a fluorescence microscope (BX51; Olympus).

#### Terminal deoxynucleotidyl transferase-dUTP nick-end labeling (TUNEL) staining

Apoptosis was assessed in paraffin-embedded xenograft sections of 5  $\mu$ m in thickness, collected at the end of treatment experiment. TUNEL staining was performed by using the In Situ Cell Death Detection Kit (TMR red; Roche Applied Science) according to the manufacturer's procedure. Slides were imaged and analyzed using Image J 1.43 freeware (NIH) and were blinded to the analyzer. Apoptosis was





**Figure 4.** Effects of apatinib and CPT-11 on the expression of VEGF, VEGFR2 and MMP9 in the PDX model of CRC. Magnification:  $\times 200$ ; scale bar, 100  $\mu\text{m}$ .

quantified by scoring the percentage of TUNEL-positive cells. Apoptotic versus total cells were counted in at least five randomly chosen microscopic fields (magnification 40  $\times$ ) and 500 total cells.

#### Western blotting

Western blotting was performed with a SDS-PAGE electrophoresis system. Briefly, protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). After blocking with 5% nonfat milk, membranes were incubated overnight at 4°C with primary antibodies against AKT (1:1000), phospho-AKT (1:1000), ERK (1:1000) and phospho-ERK (1:1000). Primary antibodies were all purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies were detected with an enhanced chemiluminescence detection system (Millipore, Billerica, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000, Santa Cruz, CA) was used as a loading control.

#### Enzyme linked immuno sorbent assay (ELISA)

Blood was obtained from mice at sacrifice and serum was collected by routine procedure. The serum levels of IL-6, IL-8 and TNF- $\alpha$  were mea-

sured using the specific ELISA kits (R&D Systems) following the manufacturer's instructions. All experiments were conducted in duplicate.

#### Statistical analysis

Statistical analyses were performed with mean  $\pm$  SD. Differences between groups were assessed using one-way analysis of variance (ANOVA). Statistical calculations were performed using GraphPad Prism software version 5 (GraphPad Software, Inc.), and  $P < 0.05$  was considered statistically significant.

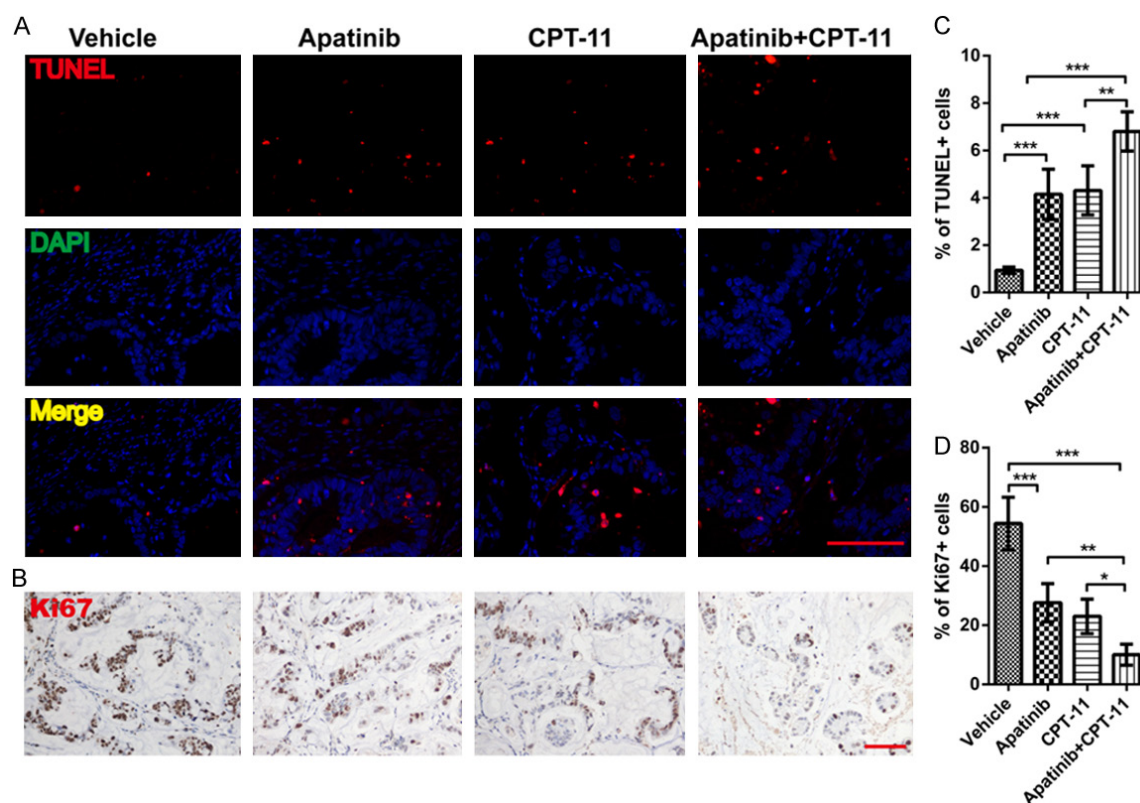
## Results

#### Sequencing analysis of KRAS, NRAS and BRAF mutations

Mutational analysis of the KRAS, NRAS and BRAF genes was performed for xenograft tumors from CRC. KRAS gene exhibited GG-T $\rightarrow$ GTT missense mutation in codon 12 of exon 2 (**Figure 1A**), while no other mutations of NRAS in exon 2 and BRAF in exon 15 were found (**Figure 1B, 1C**).

#### Antitumor activity of apatinib in combination with CPT-11

We next evaluated the antitumor activity of apatinib in combination with CPT-11 in the PDX



**Figure 5.** Apatinib in combination with CPT-11 significantly increased apoptosis and decreased proliferation in the PDX model of CRC. Tumors harvested at the end of each in vivo treatment study were examined for apoptosis by TUNEL analysis and cell proliferation by IHC for Ki67. A. Representative images of TUNEL-stained (red) sections with DAPI counterstain (blue) are shown. Magnification:  $\times 400$ ; scale bar, 100  $\mu\text{m}$ . B. Representative images of proliferative cells stained with Ki-67. Magnification:  $\times 200$ ; scale bar, 100  $\mu\text{m}$ . C. Blinded quantitative analysis of Ki67 as described in Materials and Methods. D. Blinded quantitative analysis of apoptotic cells, as determined by TUNEL, as described in Materials and Methods. Bars, mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

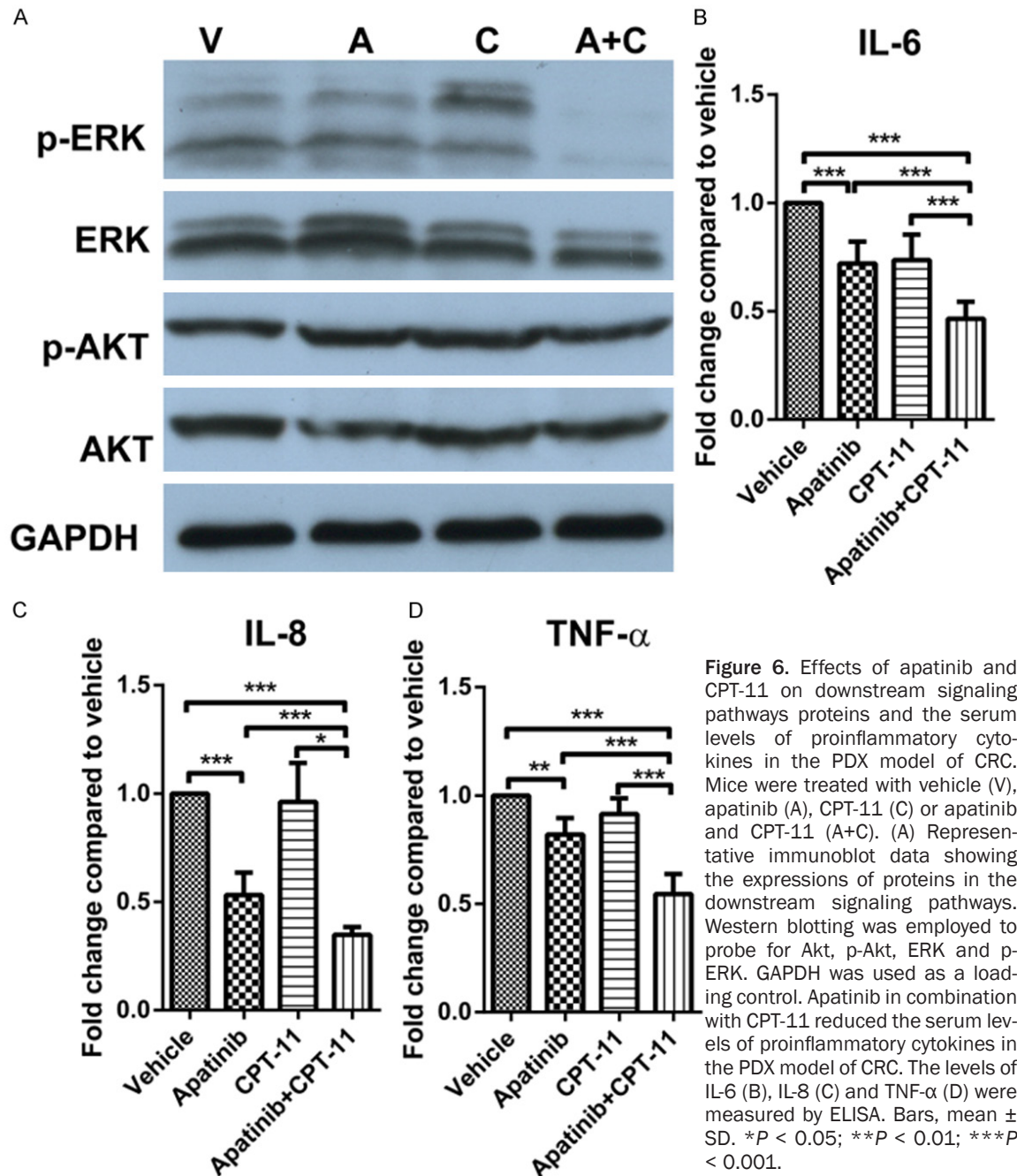
model of CRC in vivo. Apatinib was orally administered at 150 mg/kg daily and CPT-11 was administered i.v. at 30 mg/kg twice a week for 21 d. On day 22 (21 d after the treatment was initiated), our results showed that single agent treatment in the PDX model reduced tumor growth by 60.8% (apatinib,  $P < 0.001$ ) and 48.2% (CPT-11,  $P < 0.001$ ) compared with vehicle, and an even more significant effect on tumor growth was observed in combination treated groups (82.1% reduction with apatinib and CPT-11,  $P < 0.001$ ) (**Figure 2A**). At the end of treatment, there were also statistically significant differences in tumor volume and weight between the group treated with apatinib in combination with CPT-11 and the groups treated with apatinib and CPT-11 alone in the PDX model (**Figure 2B, 2C**). Overall, all treatments were well tolerated. No obvious side effects or weight loss was observed during the experimental period of either xenograft model (**Figure 2D**).

#### Effect of apatinib in combination with CPT-11 on tumor-associated angiogenesis

To examine the effect of apatinib in combination with CPT-11 on tumor-associated angiogenesis in tumor, selected tumors were stained with CD31 and  $\alpha\text{SMA}$  antibody, so that the vasculature could be visualized. This analysis revealed that vasculature density significantly decreased in apatinib combined with CPT-11 treated xenografts compared to vehicle treated group, while scarcely decreased in single CPT-11 treated group (**Figure 3**).

By immunohistochemical staining, we found that VEGFR2 and MMP9 expressions were significantly suppressed in apatinib in combination with CPT-11 treated group, but not for VEGF. VEGFR2 expressions in single apatinib treated group was also significantly suppressed (**Figure 4**). These results indicated that the combination of apatinib and CPT-11 inhibited





**Figure 6.** Effects of apatinib and CPT-11 on downstream signaling pathways proteins and the serum levels of proinflammatory cytokines in the PDX model of CRC. Mice were treated with vehicle (V), apatinib (A), CPT-11 (C) or apatinib and CPT-11 (A+C). (A) Representative immunoblot data showing the expressions of proteins in the downstream signaling pathways. Western blotting was employed to probe for Akt, p-Akt, ERK and p-ERK. GAPDH was used as a loading control. Apatinib in combination with CPT-11 reduced the serum levels of proinflammatory cytokines in the PDX model of CRC. The levels of IL-6 (B), IL-8 (C) and TNF- $\alpha$  (D) were measured by ELISA. Bars, mean  $\pm$  SD. \* $P$  < 0.05; \*\* $P$  < 0.01; \*\*\* $P$  < 0.001.

tumor growth by decreasing microvessel density, and the antiangiogenic effects were mainly induced by apatinib not by CPT-11.

#### *Effects of apatinib in combination with CPT-11 on cell apoptosis and proliferation*

To analyze the effects of treatment with apatinib, CPT-11, and combined treatment on cell apoptosis and proliferation, selected tumors were subjected to TUNEL analysis (Figure 5A)

as well as Ki67 IHC (Figure 5B). Significantly increased TUNEL staining was found in the group treated with apatinib with and without CPT-11, compared with vehicle treated group (Figure 5C). Significantly decreased expression of Ki67 was also observed in tumors treated with apatinib, CPT-11 alone or the combination of apatinib and CPT-11, compared with vehicle treated group (Figure 5D). These results suggested that the combination of apatinib and CPT-11 inhibited tumor growth by increas-

ing cell apoptosis and decreasing cell proliferation.

### *Effect of apatinib in combination with CPT-11 on downstream signaling pathways proteins*

To examine the effect of combination treatment on downstream signaling pathways proteins, we measured the protein levels of AKT, p-AKT, ERK and p-ERK in xenografts harvested at the end of each treatment group by using western blotting. No significant differences were found in each group of the PDX model, except that the expression level of p-ERK was downregulated after combination therapy with apatinib and CPT-11 (**Figure 6A**).

### *Combination treatment with apatinib and CPT-11 decreased the proinflammatory cytokines*

Proinflammatory cytokines may be an underlying factor promoting tumor progression by stimulating processes such as angiogenesis, invasion, and metastasis. To examine the effect of combination treatment on proinflammatory cytokines, we measured the serum levels of IL-6, IL-8 and TNF- $\alpha$  by using ELISA. In this study, Treatment with apatinib and CPT-11 resulted in significant reduction in secretions of IL-6 (46.5%), IL-8 (34.8%) and TNF- $\alpha$  (54.6%), compared with vehicle treated group (**Figure 6B-D**).

## Discussion

In the last decade, the introduction of targeted therapeutic options, such as anti-angiogenic drugs and anti-EGFR monoclonal antibodies, has significantly improved the overall survival of patients with metastatic CRC [17, 37]. The mutational status of KRAS is used as the validated marker of response to anti-EGFR antibodies [4]. Treatment of metastatic CRC patients with KRAS mutation with anti-angiogenic drugs, such as bevacizumab and regorafenib, has been shown to induce significant clinical benefit and represents standard of care [19, 38]. However, the benefit of anti-angiogenic drugs in clinical therapy is marginal and transient, inevitably tumor develops drug resistance [23, 39]. Therefore, development of new treatment strategies is urgently required.

Apatinib (YN968D1) is a novel highly selective inhibitor against VEGFR2, and it is currently

being evaluated in clinical trials in a number of tumor types [27, 28]. Recently, apatinib demonstrated a significant improvement in overall survival in patients with metastatic gastric cancer who failed previous therapies, and has subsequently become the first small-molecule VEGFR inhibitor approved treatment for metastatic gastric cancer by the China Food and Drug Administration [26]. However, the effect of apatinib in preclinical model of CRC has yet to be investigated.

In the present study, we successfully established one patient-derived xenograft model, which was obtained from a liver metastasis of patient with CRC developed during anti-EGFR treatment. First, to investigate the molecular characteristics and identify potential therapeutic targets of the PDX model, we used direct sequencing to identify the most frequent mutations of KRAS, NRAS and BRAF. Our data showed that the PDX model harbored mutation-type for KRAS and wild-type for NRAS and BRAF, which was in consistent with the original patient tumor tissues. We had previously described that serially passaged PDXs could retain the molecular characteristics of origin [33].

Then, we evaluated the antitumor activity of apatinib alone or in combination with CPT-11 in vivo model of CRC. Our in vivo study using PDX model of KRAS-mutant colorectal cancer demonstrated that apatinib in combination with CPT-11 effectively inhibited tumor growth without affecting body weight. We also found that combination treatment reduced the expression levels of angiogenesis-related protein such as VEGFR2 and metastasis-related protein such as MMP9. Additionally, combination treatment decreased proinflammatory cytokines in vivo. Further data analysis showed that combination treatment reduced microvessel density, suppressed proliferation, and increased apoptosis compared to either apatinib or CPT-11 alone, indicating that the observed synergistic effect might be attributed to the dual inhibition on tumor signaling and tumor microenvironment. To our knowledge, this is the first report in which the combination of VEGFR2 inhibitor and cytotoxic drug has shown synergistic antitumor effects in vivo preclinical colorectal cancer models.

The molecular mechanisms of the synergistic effect apatinib and CPT-11 in the xenografts



remain to be elucidated. One explanation is the potential vascular normalization effect of apatinib-mediated in solid tumors as we are investigating now in our group, which may enhance delivery of cytotoxic drug, such as CPT-11. Another explanation is that metronomic dosing of CPT-11 treatment can cause changes in tumor-associated blood vessels that are comparable to those observed when using anti-angiogenic drugs [40]. However, in the present study, our data demonstrated that there was no significant decrease in microvessel density after treatment with CPT-11 compared with vehicle treatment. It is unclear why the effect occurs, and further studies should be done to illuminate the real reasons, which may help to improve and optimize the therapeutic results for the combination treatment with apatinib and CPT-11.

In conclusion, we demonstrated that a rational combination strategy using apatinib and CPT-11 was active against metastatic CRC. Further clinical trials are required to evaluate this beneficial combination as a potential treatment strategy for metastatic CRC patients.

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## Disclosure of conflict of interest

None.

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