Cabozantinib, a Novel c-Met Inhibitor, Inhibits Colorectal Cancer Development in a Xenograft Model

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Angiogenesis plays a critical role during tumor development. c-Met has recently been implicated in the angiogenesis of various tumors, leaving its role in colorectal cancer (CRC) unknown. In this study, we aimed to evaluate the effect of a novel c-Met inhibitor, cabozantinib, on the tumor growth and angiogenesis in a CRC mouse model.

Material/Methods: A mouse CRC xenograft model was used to evaluate the effect of cabozantinib on *vivo* growth of tumors and angiogenesis. The expression of angiogenesis-related factors was evaluated by immunohistochemistry (IHC) and Western blotting. Levels of serum cytokines were detected by ELISA.

Results: Cabozantinib effectively reduced tumor size and angiogenesis, and suppressed the expression of vascular endothelial growth factor (VEGF) in tumor tissues, possibly via the inhibition of Sonic Hedgehog (SHH) pathway.

Conclusions: The blockade of c-Met inhibits the tumor growth and angiogenesis via modulating SHH pathway, suggesting a potential strategy in the treatment of CRC.

MeSH Keywords: Colorectal Neoplasms • Colorectal Neoplasms, Hereditary Nonpolyposis • Technetium Tc 99m Medronate

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Background

Colorectal cancer (CRC) is the second leading cause of mortality among all malignant tumors worldwide [1]. Surgical resection remains the primary management for CRC. However, more than 25% of patients with complete resection develop local recurrence or distant metastasis within 5 years [2].

Angiogenesis plays a critical role in the development of solid tumors [3]. Sonic Hedgehog (SHH) pathway is a key cellular signaling pathway regulating tumor angiogenesis, as aberrant activation of SHH signaling pathway has been implicated in many cancers [4]. The activation of SHH pathway requires the participation of transmembrane receptor Patched-1 (PTCH-1) and smoothened (Smo) that regulates the expression of vascular endothelial growth factor A (VEGF-A) [5,6], which is believed to be one of the most powerful stimulators of angiogenesis [7]. c-Met is a tyrosine kinase receptor that acts as a proto-oncogene and is activated by hepatocyte growth factor [8]. The activation of c-Met can facilitate tumor cell motility, proliferation, invasion, and survival [9]. The HGF/c-Met pathway is activated in various solid tumors, including breast carcinoma, glioblastomas, and ovarian cancer [9,10] and correlates with unfavorable prognosis [11–13], or tumor aggressiveness and chemotherapy resistance [14]. c-Met activation can drive lymphangiogenesis [15], leading to lymph node metastasis. Recent data have also shown that c-Met suppresses cell proliferation and angiogenesis of gastric cancer [16]. The role of c-Met in CRC, however, remains to be illustrated. We thus hypothesized that the inhibition of c-Met would attenuate the tumorigenesis of CRC via the suppression of SHH signaling pathway by using a CRC mouse model.

Material and Methods

Cell culture

Human CRC cell line HT-29 is a product of the American Type Culture Collection (ATCC, USA) and was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (v/v, Hyclone) in a 37°C humidified incubator containing 5% CO2.

Mice

Athymic BALB/c nu/nu male mice (20–25 g) were obtained from Beijing Vital Rivers Laboratory Animal Co., Ltd. (Beijing, China) and were kept in a specific pathogen-free (SPF) facility with a 12-h light/dark cycle. All animal treatments were in accordance with international ethics guidelines and the National Institutes of Health Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Care and Use Committee of Jinan Military General Hospital.

Induction of CRC xenograft model

Human HT-29 cells were grown in medium as described above. Cells were detached by trypsinization, followed by washing, and re-suspended in serum-free DMEM. We mixed 1.5×10^6 cells with Matrigel (1:1) and subcutaneously injected it in the right flank of athymic nude mice to induce tumor growth.

Drug treatments

Six days after tumor cell implantation, 24 mice were randomly divided into control and treatment groups (n=12 per group). Cabozantinib (XL184, South San Francisco) was re-suspended in water and given daily by oral gavage at a dose of 100 mg/kg in a volume of 100 μl. The control group received equal volume of water by oral gavage [17]. Drug treatments lasted for 18 days (from day 6 after HT-29 cell injection until day 24).

Body weight and tumor size

Body weight and tumor size were measured every other day using an electronic balance and a vernier caliper. Tumor volume was calculated using the formula:

$$volume = 0.5 \times length \times width \times depth.$$

Tissue sample collection

After the endpoint (24 days after the implantation), blood was collected from the heart and the serum was separated and frozen at -20°C for further analyses. Mice were then sacrificed for the collection of tumors. Tissue samples were frozen in liquid nitrogen and stored at -80°C for protein extraction.

Immunohistochemistry (IHC)

Tumor samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned into 4-μm-thickness slices. After dewaxing, tissues sections were processed by antigen retrieval, followed by the quenching of endogenous peroxidase activity using hydrogen peroxide. PBS (0.1% Tween 20) containing normal serum was used to block non-specific binding sites. Slides were incubated with rabbit polyclonal antibodies against CD31 or VEGF-A (in 1:50 dilution, Santa Cruz, US). After washing with PBS, slides were incubated with biotinylated anti-rabbit IgG antibody followed by horseradish peroxidase-conjugated streptavidin (Sigma, US). After developing in DAB substrates (Invitrogen, US), 5 high-magnification fields (400×) were randomly selected in each slide, and the numbers of positive cells in each field were counted by the image analysis system (Image-Pro Plus). In a parallel negative control group, PBS was applied to replace the primary antibody.
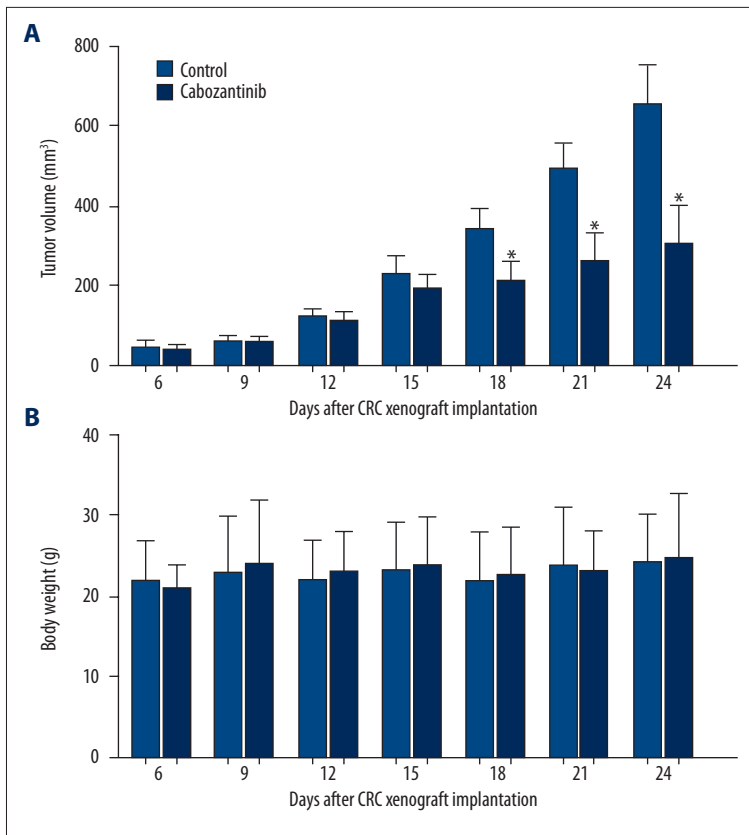
Figure 1. Effect of cabozantinib on tumor growth in colorectal cancer (CRC) mice. After tumor development, the mice were given a dose of 100 mg/kg cabozantinib by oral gavage for 18 days. Tumor volume (**A**) and body weight (**B**) were measured during the experiment. Data shown are mean ±SD from 12 individual mice in each group. * $p < 0.01$, versus controls.

Western blotting

To determine the molecular mechanism of cabozantinib on SHH signaling pathway, Western blot analysis was performed to detect key proteins involved in SHH pathway. Proteins (50 μg) were extracted from CRC tissues, resolved by 8% SDS-PAGE, transferred onto PVDF membranes, and detected by specific antibodies against SHH, PTCH-1 or SMO using chemiluminescence system detection kit (Qiagen).

ELISA

The serum levels of TNF-α, IL-1β and IL-6 were measured using the specific ELISA kits (R&D Systems) following the manufacturer's instructions. All experiments were conducted in duplicate.

Statistical analysis

All collected data are presented as mean ±SD, unless otherwise specified. Data were tested for normality by using K-S test. One-way ANOVA followed by post-hoc comparisons was used to evaluate differences among the 3 groups in tumor weight and serum indexes. All analyses were performed using SPSS 17.0 software. A statistical significance was defined when $p < 0.05$.

Results

Cabozantinib inhibited tumor growth

The therapeutic efficacy of cabozantinib was firstly evaluated by its inhibition on the tumor volume of CRC xenograft, in addition to its adverse effects on body weight. As shown in Figure 1A, cabozantinib treatment significantly reduced the tumor size compared to control ones ($p < 0.05$). The drug also did not affect body weight (Figure 1B). These data collectively suggest the potency of cabozantinib in suppressing *in vivo* growth of CRC, without significant adverse effects.

Cabozantinib inhibited tumor angiogenesis

Because angiogenesis plays an important role in the progression and metastasis of cancers, we examined the effect of cabozantinib on intratumoral microvessel density (MVD), using the endothelial cell-specific marker CD31. As shown in Figure 2A, the percentage of CD31-positive cells in cabozantinib-treated mice was significantly reduced compared to control animals ($p < 0.05$). As one of the most powerful angiogenesis stimulators, the expression of VEGF-A was also suppressed by cabozantinib treatment (Figure 2B). These results illustrate the inhibition of angiogenesis of CRC by cabozantinib.
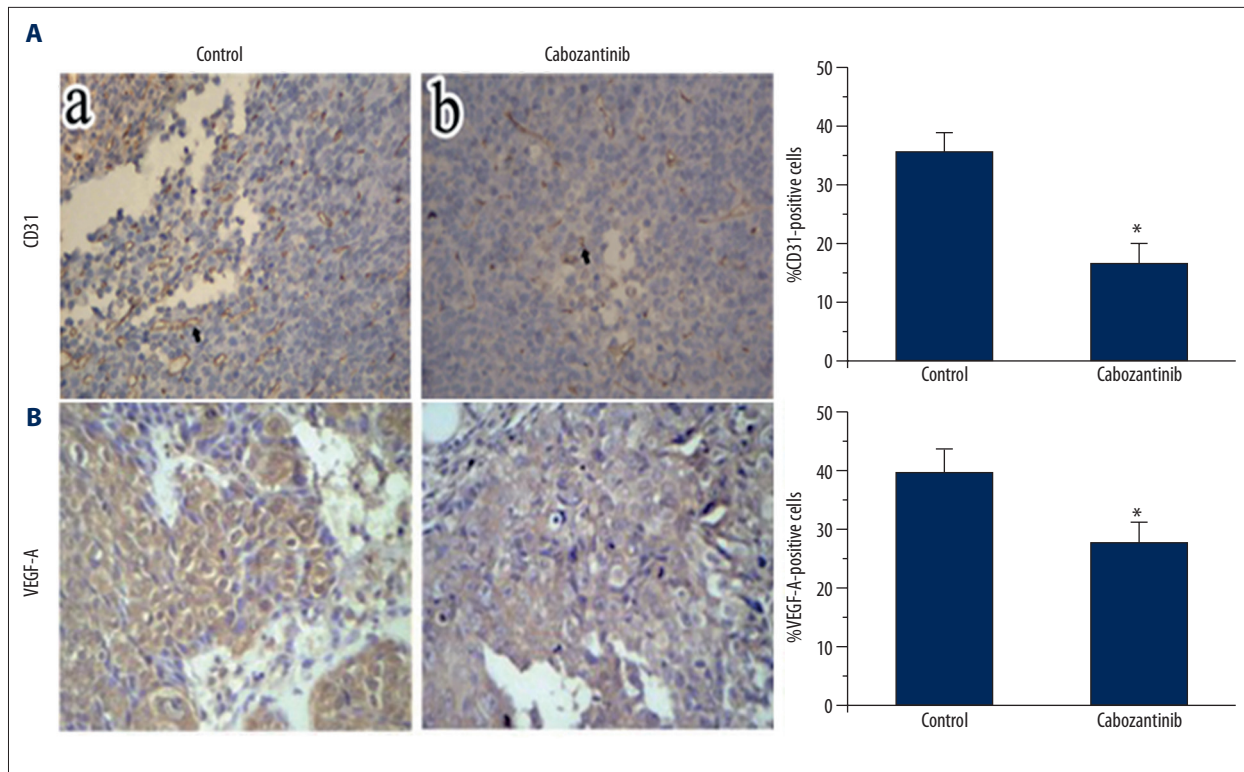
Figure 2. Cabozantinib treatment reduced intratumoral microvessel density (MVD) in colon cancer model. Representative areas from tumors of mice showing CD31 (**A**) and VEGF (**B**) staining. The photographs were representative images at a magnification of ×400. Semi-quantification of IHC is expressed as percentage of positive-staining cells. Data are mean ±SD from 10 mice in each group.* p<0.05, versus controls. Magnification ×200.

Cabozantinib suppressed SHH signaling pathway

Tumor angiogenesis is believed to be regulated by the SHH pathway; therefore, we assessed the effect of cabozantinib on the expression of key mediators in the SHH pathway using Western blot analysis. As shown in Figure 3, cabozantinib treatment significantly reduced the protein level of SHH, PTCH-1 and SMO in tumor samples. Collectively, these data indicate that the inhibitory role of cabozantinib in tumor angiogenesis is possibly mediated by its suppression on the SHH pathway.

Cabozantinib treatment decreased the proinflammatory cytokines

Tumor development after chronic inflammation is typically dependent on the production of inflammatory cytokines that can recruit immune cells and promote the production of mutagenic factors. This study found dramatic elevation of inflammatory cytokines, including TNF-α, IL-6 and IL-1β in control mice, while cabozantinib treatment substantially reduced the production of these cytokines (Figure 4).

Discussion

In this study we examined the effects of a novel c-Met antagonist, cabozantinib, on the *in vivo* growth of CRC and explored its possible underlying mechanisms. Our data demonstrated that cabozantinib effectively inhibited tumor growth without affecting body weight. We also found that cabozantinib reduced the expression levels of angiogenesis-related proteins, including CD31 and VEGF. Further data showed that the SHH signaling pathway was significantly suppressed in cabozantinib-treated mice. Additionally, cabozantinib treatment decreased pro-inflammatory cytokines *in vivo*. Taken together, these findings indicate that c-Met antagonism may be beneficial against the progression of CRC by suppressing angiogenesis via inhibition of the SHH signaling pathway in CRC.

Angiogenesis plays a crucial role in the progression of CRC, as proved by the high expression of HMGB1, which is an angiogenesis-related nuclear factor [18]. c-Met overexpression promotes tumor angiogenesis, thereby contributing to reduced survival [19]. Studies have indicated that HGF/c-Met acts in an alternative angiogenesis pathway in drug-resistant tumors [20]. It is also reported that c-Met is expressed in endothelial cells, and HGF, a c-Met ligand, can stimulate the
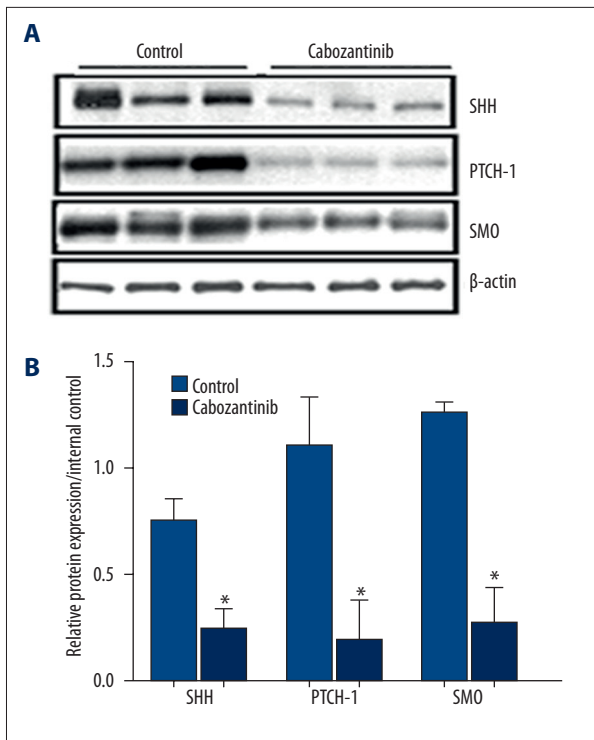
Figure 3. Effect of cabozantinib on the activation of Sonic Hedgehog (SHH) pathway in CRC xenograft mice. Tumor tissues were processed for Western blotting for SHH, PTCH-1, and SMO. Representative images were taken (**A**) and quantification of Western blot assay is represented as percentage of internal control (**B**). Data are mean ±SD. * p<0.01, versus controls.

our results suggest that the inhibition of c-met by cabozantinib suppressed angiogenesis *in vivo*, suggesting a potential mechanism by which cabozantinib exerts its anti-CRC effects.

The SHH pathway induces the expression of angiopoietins and VEGF from mesenchymal cells, highlighting the significance of SHH signaling in tumor angiogenesis [23]. While most of anti-angiogenic research has focused on inhibition of VEGF, the efficacy is somewhat limited, mainly due to inadequate alternate targets and paucity of selective drugs [24]. The survival of drug-resistant tumor cells is favored by the fact that there is redundancy of the pathways and growth factors in tumor angiogenesis. Our study for the first time showed that c-met inhibition by cabozantinib can inactivate the SHH pathway *in vivo*, suggesting that the suppression of angiogenesis is partially due to SHH inhibition. In addition, because chronic inflammation has been recognized as a promoter of colon carcinogenesis [25], our finding that cabozantinib-treated mice exhibited significantly lower levels of the proinflammatory cytokines, including TNF-α, IL-6, and IL-1β, may provide an alternative explanation for its efficacy in tumor treatment.

Conclusions

This study demonstrated a crucial role of c-Met during tumorigenesis, especially tumor angiogenesis, of CRC. The blocking of c-Met attenuated CRC tumor growth and angiogenesis *in vivo*. Therefore, c-Met may be a potential drug target for treating CRC patients, although its detailed cellular pathways need to be further established

Conflict of interest

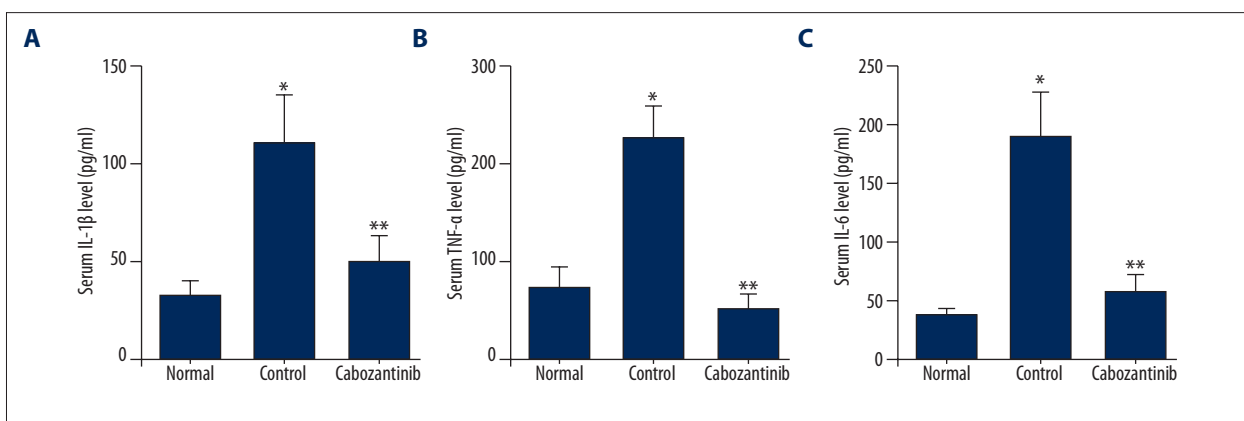All authors declare no conflicts of interest in this study.

growth, invasion, and motility of cells [21]. In addition, endothelial cells with higher expression of c-met were more sensitive to HGF and exhibited a higher rate of abnormal morphology. This role received further support from the fact that the antibody against the extracellular domain of c-met inhibited angiogenesis *in vitro* [22]. Consistent with these findings,

Figure 4. Cabozantinib reduced the serum levels of cytokines in CRC xenograft mice. The serum levels of IL-1β (**A**), TNF-α (**B**) and IL-6 (**C**) were measured by ELISA. CRC xenograft mice showed higher levels and treatment with cabozantinib reduced their levels. Data are expressed as mean ±SD, * p<0.01, compared to normal controls, ** p<0.01 compared to control-treated controls.

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