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Bevacizumab-enhanced antitumor effect of 5-fluorouracil via upregulation of thymidine phosphorylase through vascular endothelial growth factor A/vascular endothelial growth factor receptor 2-specificity protein 1 pathway

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Bevacizumab (Bv) can be used synergistically with fluoropyrimidine-based chemotherapy to treat colorectal cancer. Whether and how it affects the delivery of fluoropyrimidine drugs is unknown. The present study aimed to explore the effect of Bv on the delivery of 5-fluorouracil (5-FU) to tumors and the underlying mechanism from metabolic perspective. Bv enhanced the anti-tumor effects of 5-FU in LoVo colon cancer xenograft mice and increased the 5-FU concentration in tumors without affecting hepatic 5-FU metabolism. Interestingly, Bv remarkably upregulated thymidine phosphorylase (TP) in tumors, which mediated the metabolic activation of 5-FU. Although TP is reported to promote angiogenesis and resistance, the combination of Bv and 5-FU resulted in anti-angiogenesis and vessel normalization in tumors, indicating that the elevated TP mainly contributed to the enhanced response to 5-FU. Bv also induced TP upregulation in LoVo cancer cells. Treatment with vascular endothelial growth factor receptor 2 (VEGFR2) antagonist apatinib and VEGFR2 silencing further confirmed TP upregulation. Bv and apatinib both enhanced the cytotoxicity of 5-FU in LoVo cells, but there was no synergism with adriamycin and paclitaxel. We further demonstrated that the effect of Bv was dependent on VEGFR2 blockade and specificity protein 1 activation via MDM2 inhibition. In summary, Bv enhanced the accumulation of 5-FU in tumors and the cytotoxicity of 5-FU via TP upregulation. We provide data to better understand how Bv synergizes with 5-FU from metabolic perspective, and it may give clues to the superiority of Bv in combination with fluoropyrimidine drugs compared to other chemotherapeutic drugs in colon cancer.

Abbreviations: Bv, bevacizumab; CRC, colorectal cancer; DPD, dihydropyrimidine dehydrogenase; 5-FU, 5-fluorouracil; 5-FUH_2, 5,6-dihydro-5-fluorouracil; Ip, intraperitoneal injection; MDM2, murine double minute-2; Q-PCR, Quantitative real-time PCR; TK, thymidine kinase; TP, thymidine phosphorylase; TS, thymidylate synthase; VEGFA, vascular endothelial growth factor A.

Liu and Zhang Contributed equally to this work.

KEYWORDS
5-fluorouracil, bevacizumab, chemosensitization, thymidine phosphorylase, VEGFA/VEGFR2 blockade

1 | INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the 4th most common cause of cancer deaths worldwide.[1] Bevacizumab (Bv), the first recombinant humanized vascular endothelial growth factor (VEGF) monoclonal antibody approved by the FDA, has synergistic benefits for CRC when used with chemotherapy. Bv may be preferred over chemotherapy regimens, including FOLFIRI (fluorouracil and folinic acid with irinotecan),[2,3] XELIRI (capecitabine plus irinotecan)[2] and cisplatin-paclitaxel[4] treatment. The National Comprehensive Cancer Network (NCCN) guidelines recommend the use of Bv with first-line or second-line chemotherapy regimens in CRC treatment. Some studies ascribe Bv's synergism for chemotherapeutics to its anti-angiogenic activities[5,6] or the tumor vascular normalization during a certain time period.[7] In addition, Bv improves the tumor delivery of chemotherapeutic drugs, such as topotecan and etoposide.[8,9] Whether Bv can exert a similar effect on the behavior of fluoropyrimidine drugs in tumors remains unknown.

Since the 1950s, 5-fluorouracil (5-FU) has been the first-line therapy for CRC.[10] Due to the complex multi-step enzymatic conversion of 5-FU, its metabolism can be easily influenced by another co-administered drug. Four intracellular enzymes are key determinants in 5-FU metabolism: dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP), thymidine kinase (TK) and thymidylate synthase (TS). More than 80% of administered 5-FU is reduced to 5,6-dihydro-5-fluorouracil (5-FUH_2) by DPD in the liver[10,11] to be inactivated. In tumor tissues, 5-FU could be transferred to its activated form and finally affect DNA synthesis. 5-FU is first converted to 5-fluoro-2′-deoxyuridine (FdUrd) by TP, which is overexpressed in many types of cancer.[12,13] Then, FdUrd is phosphorylated by TK to FdUMP, which directly binds to TS and inhibits its activity on DNA synthesis.[14] In addition, uridine phosphorylase (UP), uridine kinase (UK) and orotate phosphoribosyltransferase (OPRT) can covert 5-FU to FUMP and cause RNA synthesis inhibition. The present study innovatively explored the effects of Bv on the multi-step metabolism and tumor delivery of 5-FU in CRC models.

Among the above enzymes, TP plays a dual role in cancer. As TP converts 5-FU to its active metabolite in the first step, the overexpression of TP in cell culture and xenograft models had been shown to increase sensitivity to 5-FU due to enhanced formation of FdUMP. Interestingly, in contrast to the key role in determining fluoropyrimidine response in chemotherapy, TP is also identical to the angiogenic factor platelet-derived endothelial cell growth factor (PD-ECGF) and can aid cancer angiogenesis and progression.[15,16] TP catalyzes phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate, which is further converted to 2-deoxy-D-ribose, a downstream mediator of TP. Then, 2-deoxy-D-ribose can induce the production of VEGFA, binding to VEGF receptor (VEGFR) to promote angiogenesis.[17] Because Bv neutralizes VEGFA, whether Bv can influence TP expression via a feedback mechanism after the VEGFA/VEGFR pathway blockade is not known. We thus studied the underlying mechanisms of Bv's synergistic effect on 5-FU from a drug metabolic perspective. These data may help us better understand and treat CRC.

2 | MATERIALS AND METHODS

2.1 | Animals and treatments

Male athymic BALB/c nude mice (9 weeks old, 18-22 g) were purchased from Shanghai Slack Laboratory Animal (Shanghai, China). The mice were maintained in autoclaved filter-top microisolator cages in a temperature-controlled environment and were provided with food and water *ad libitum*. Animal care and surgery protocols were approved by the Animal Care Committee of China Pharmaceutical University. First, mice were randomized to treatment groups equally: (i) control (n = 8): saline (10 mL/kg/d, ip); (ii) 5-FU (n = 8): 15 mg/kg/3 days, ip; (iii) Bv (n = 8): 5 mg/kg/3 days, ip; and (iv) 5-FU+ Bv (n = 8): BV 5 mg/kg/3 days, ip; 5-FU 15 mg/kg/3 days, ip. Animals were treated from days 0 to 14. At day 15, mice were given Bv or 5-FU followed by venous blood collection and then killed at a pre-set time point.

2.2 | Studies of xenograft tumor growth and surgery for tumor tissues

For tumor growth studies, mice were weighed and the major axis (*a*) and minor axis (*b*) of tumors were measured daily until day 14 before and during treatment. Tumor volume (TV) was calculated using the following formula: TV (mm^3) = $1/2 \times a \times b^2$. At day 15, mice were killed, and tumor xenografts were removed and weighed. Tumor inhibition was calculated using the following formula: Inhibition rate = (1 − mean volume of treatment group/mean volume of control group) × 100%. The removed tumor was further divided into 3 pieces, and 2 pieces were rapidly frozen in drikold and stored at −80°C for drug concentration determination and molecular biological assays. Other tissues were fixed in 4% PFA for immunostaining.

2.3 | Supplementary materials and methods

Details on the measurement of 5-FU concentration and metabolism, enzyme expression and morphological changes of tumor microvessels and are provided in Appendix S1.

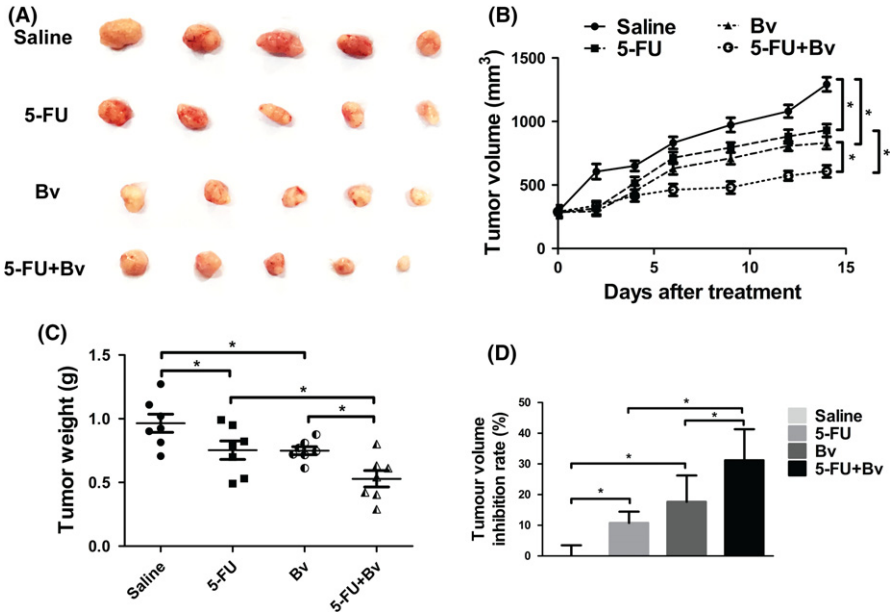

FIGURE 1 Effects of bevacizumab (Bv) with/without 5-fluorouracil (5-FU) on tumor growth in LoVo colon cancer xenograft mice. Mice were intraperitoneally injected with 5-FU (15 mg/kg/3d), with or without Bv (5 mg/kg/3d) for 15 days. A, Tumors excised on day 15. B, Tumor growth curves during treatment. C, Tumor weights from all groups on day 15. D, Tumor volume inhibition rates at day 14. *$P < 0.05$, n = 8

2.4 | Statistical analysis

All data are presented as means ± SEM. Statistical analysis was performed using one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

3 | RESULTS

3.1 | Bevacizumab-enhanced chemotherapeutic efficacy of 5-fluorouracil on tumor growth in LoVo colon cancer xenograft mice

The efficacy of the combined treatment of 5-FU and Bv on LoVo colon cancer growth was evaluated. Tumor growth curves showed that the tumors of the combination treatment group grew slower than those of the 5-FU or Bv groups (Figure 1A,B). The average tumor weight of the control group was 0.96 ± 0.18 g, while other treatments significantly decreased the tumor weight, shown as 0.75 ± 0.19 g in the 5-FU group, 0.75 ± 0.08 g in the Bv group, and 0.53 ± 0.11 g in the 5-FU+Bv group, respectively (Figure 1C). As shown in Figure 1D, the combined treatment of 5-FU and Bv also exhibited higher rates of tumor inhibition (31.18 ± 5.4%) than that of the 5-FU or Bv mono-treatment group (10.80 ± 1.4% and 17.65 ± 2.6%, respectively).

3.2 | Bevacizumab increased 5-fluorouracil concentration in tumors without influence on 5-fluorouracil metabolism in liver

5-fluorouracil concentrations in plasma and tissues were determined to explore the effect of Bv on pharmacokinetic properties of 5-FU. Plasma concentration of 5-FU is shown in Figure 2A, and no obvious change had been detected between the Bv plus 5-FU group and the mono-5-FU-treated group. Interestingly, the intra-tumor concentration of 5-FU in the Bv co-treatment group increased to 1.51-fold of that in the 5-FU mono-treatment group (5.7 ± 0.6 μg/g vs 3.8 ± 1.2 μg/g, $P = 0.012$) (Figure 2B), which further confirmed the enhanced effect of Bv on 5-FU antitumor efficacy. However, Bv did not significantly affect 5-FU concentration in heart, spleen, lung, kidney and liver (Figure 2B), indicating that Bv might not enhance the toxic and side effects of 5-FU in these important organs. Because 80% of 5-FU is metabolized to 5-FUH_2 in liver, the hepatic concentration of 5-FUH_2 was determined and we found that 5-FUH_2 was not influenced by Bv (Figure 2C). The ratio of 5-FU/5-FUH_2 in the groups treated with or without Bv also exhibited no significant difference (Figure 2D). Then, the catabolic activity of DPD on 5-FU was studied in vitro. Two approaches were adopted for studying enzymatic kinetics: on one hand, 2 μmol/L 5-FU was incubated alone with S9 protein (5 mg/mL) extracted from mice liver of each group (Figure 2E), and there were no significant differences in the substrate depletion curves from the groups treated with 5-FU, Bv or the combination for 15 days; and on the other, 2 μmol/L 5-FU with or without 1 mg/mL Bv was co-incubated with S9 protein extracted from the untreated mice liver (Figure 2F), and Bv also exerted little influence on the 5-FU metabolism curve. Furthermore, Bv treated alone or jointly exhibited no significant effect on DPD gene and protein expression (Figure 2G,H), which was consistent with the data for liver S9 fraction incubation.

3.3 | Bevacizumab increased thymidine phosphorylase expression and activity in tumors

Apart from degradation by DPD, 5-FU can be converted to the active metabolites in tumor cells. On one hand, TP and TK transform 5-FU to FdUMP to bind with TS and damage DNA synthesis. On the other hand, 5-FU can be converted to FUMP by UP, UK and OPRT, hindering RNA synthesis. The results showed that Bv never affects the expression of genes related to RNA synthesis (UP, UK and

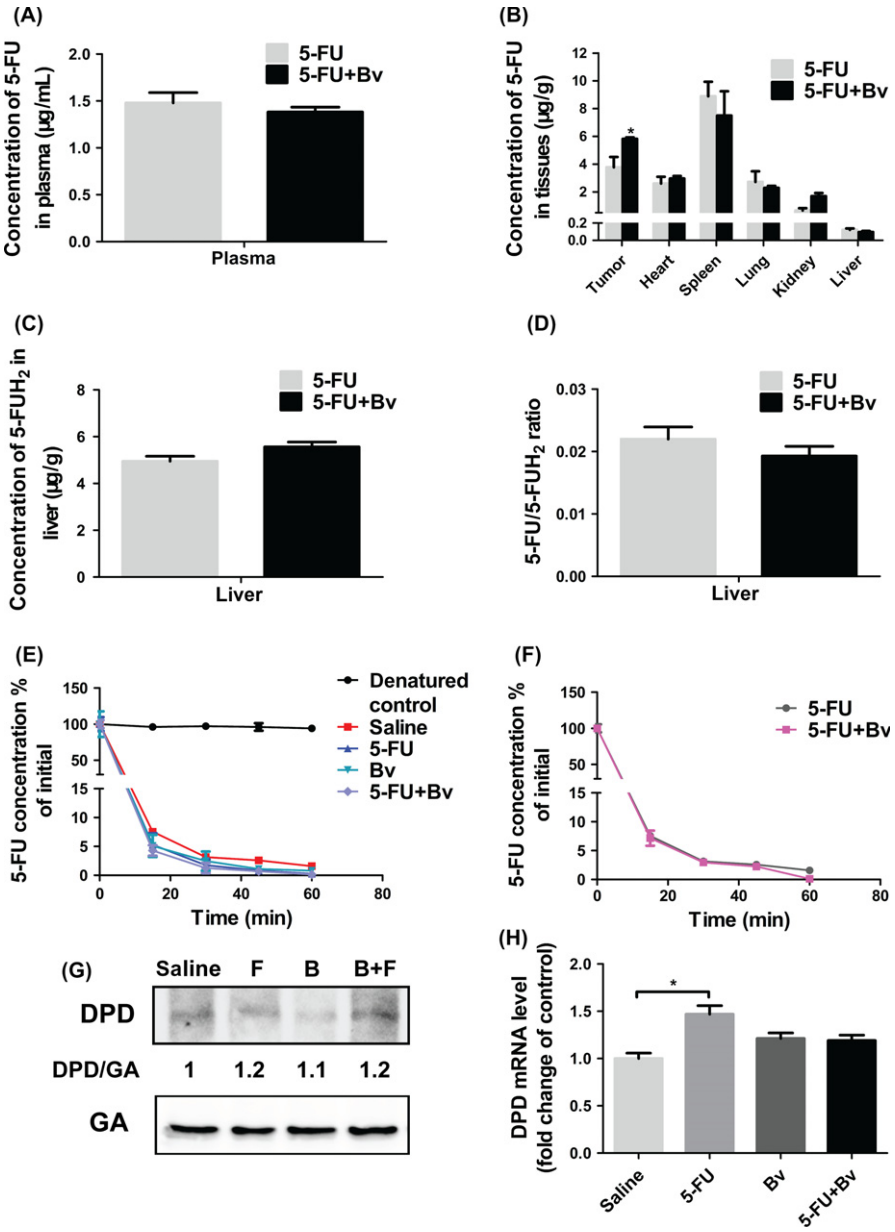

FIGURE 2 Effects of bevacizumab (Bv) on 5-fluorouracil (5-FU) pharmacokinetics and 5-FU hepatic metabolism in LoVo colon cancer xenograft mice. 5-FU concentrations were determined at 40 min after the last injection of 5-FU (15 mg/kg) on day 15. Bv (5 mg/kg) was intraperitoneally injected 60 min before administration of 5-FU. A, 5-FU concentration in plasma. B, 5-FU concentration in tumors and other tissues. C, 5-FUH_2 concentration in livers. D, Ratio of 5-FU/5-FUH_2 in livers. n = 8, $^*P < 0.05$. E and F, Depletion curves of 5-FU incubated with hepatic S9 protein mixtures. n = 6. G, Western blot for liver DPD protein; relative expression was normalized to GAPDH. n = 6. H, Q-PCR assay for liver DPD mRNA; gene expression was normalized to housekeeping gene β-actin. n = 8, $^*P < 0.05$

OPRT, Figure 3B) but significantly upregulated the expression of TP and TK at gene and protein level (Figure 3A,C). Meanwhile, we found that the expression of TP was far more than that of TK and TS in tumors. Therefore, we continued to examine Bv's effect on TP enzymatic activity, and the results showed that Bv significant reduced TP activity (Figure 3D). Our study focused on exploring the modulatory mechanism of Bv on TP expression.

3.4 | Bevacizumab reduced tumor microvessel density and promoted vascular normalization

From previous reports, we learnt that TP is identical to PD-ECGF and contributes significantly to angiogenesis, promoting tumor development. To accurately judge whether the upregulation of TP by Bv was adverse or beneficial to 5-FU chemotherapy, we tested the effect of Bv on tumor angiogenesis. First, 2-photon imaging showed that untreated and 5-FU-treated tumors displayed a chaotic network of tortuous blood vessels with a high vascular density (Figure 4A, more regions of the tumor vasculature are presented in Figure S1). However, the vascular network of the Bv-treated group showed much less excessive and chaotic branching patterns, with significantly lower vascular density (Figure 4C) and branches (Figure 4D), leading to a decrease of overall vascular volume (Figure 4B). Moving on, double staining for CD31 and the mural cell markers α-SMA showed that more pericytes covered blood vessels in Bv mono-treated or co-treated groups than in untreated and 5-FU-treated groups, indicating more mature tumor vessels (Figure 4E). Finally, we found that Bv significantly downregulated proangiogenic factors' expression and upregulated antiangiogenic factors' expression in tumors (Figure 4F,G), matching the anti-angiogenesis function of Bv. We further examined the expression of VEGFA and endostatin (changed most obviously) as well as TIMP1 (highest amount of expression) at

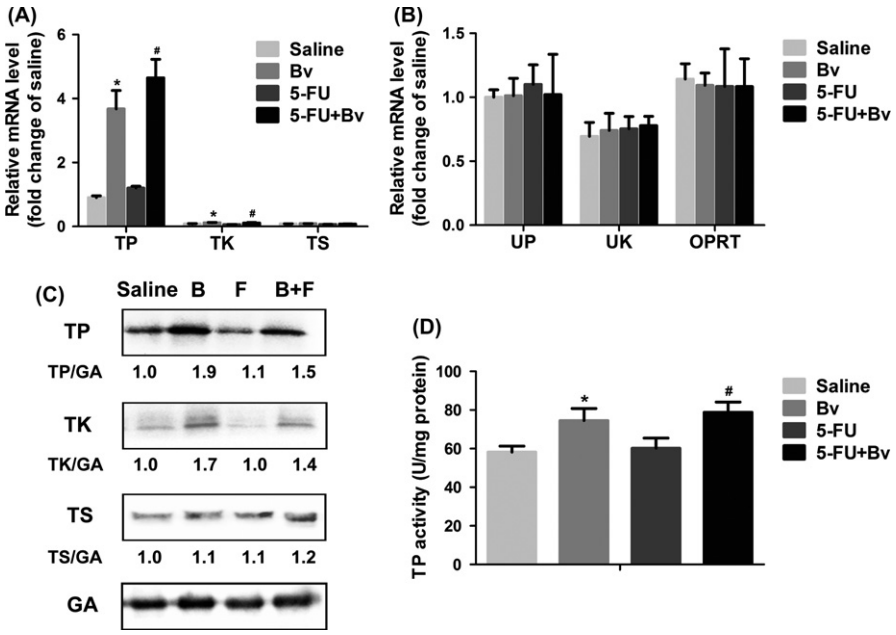

FIGURE 3 Effects of bevacizumab (Bv) on expression of 5-FU metabolic enzymes in tumors. A, Q-PCR assay for tumor TP, TK and TS mRNA. n = 8. B, Q-PCR assay for tumor UP, UK and OPRT mRNA. n = 8. C, Western blots for tumor TP, TK and TS protein. n = 6. D, The enzymatic activity of TP in tumors. $^{*}P < 0.05$ between Bv vs saline group; $^{\#}P < 0.05$ between Bv plus 5-FU group vs 5-FU group

protein level using an ELISA assay. The results showed that Bv significantly reduced the VEGFA protein level and also elevated the expression of endostatin and TIMP1 in tumor tissues, which was in accordance with the results of the gene expression assay (Figure 4H-J). These results suggested that Bv reduced tumor vascular density as well as induced tumor vessel normalization, although it upregulated TP expression.

3.5 | Thymidine phosphorylase was upregulated by inhibition of VEGFA/VEGFR2 pathway in LoVo cells

We assumed that VEGFA pathway blockade may cause a feedback upregulation on TP. LoVo cells were treated with different concentrations of Bv (1, 3, 10 μg/mL) or recombinant human VEGFA (3, 10, 30 ng/mL). As shown in Figure 5A, TP was upregulated by Bv and downregulated by VEGFA in a concentration-dependent manner. VEGFA content in cell culture medium after Bv or VEGFA treatment was detected as quality control. To further confirm the relationship between VEGFA and TP, siRNA targeting VEGFA was used. Figure 5B shows that the siRNA could silence VEGFA with high efficacy; meanwhile, the phosphorylation of VEGFR1 and VEGFR2 was remarkably blocked after VEGFA silence (Figure 5C). TP expression was upregulated by VEGFA silence, and this elevation was eliminated when recombinant VEGFA was supplemented in the medium (Figure 5D). VEGFA mainly binds to its receptor VEGFR1 and VEGFR2 to exert biological functions, so we studied whether TP was modulated by a specific VEGFR subtype. Sunitinib was chosen to antagonize VEGFR1 and apatinib to antagonize VEGFR2. IC_{50} of sunitinib was 15 nmol/L to VEGFR1 and 50 nmol/L to VEGFR2, while IC_{50} of apatinib was 70 nmol/L to VEGFR1 and 2.43 nmol/L to VEGFR2. Thus, the drug concentration for treatment was 3, 10 or 30 nmol/L sunitinib or 3, 10 or 30 nmol/L apatinib to inhibit VEGFR1 and VEGFR2, respectively. The results revealed that sunitinib hardly affected the expression of TP, while apatinib upregulated the expression of TP concentration-dependently (Figure 5E) without influence on VEGFA secretion. In addition, siRNA targeting VEGFR2 was also used for further confirmation. Efficient silencing of VEGFR2 (Figure 5G) did not affect VEGFA secretion (Figure 5F), and VEGFR2 silence elevated TP expression, which could not be reversed by VEGFA supplement.

3.6 | Bevacizumab and Apa enhanced cytotoxicity of 5-fluorouracil in LoVo cells based on thymidine phosphorylase upregulation

We further investigated whether Bv and Apa could enhance the cytotoxicity of 5-FU in vitro. Cell viability was determined by MTT assay, and the IC_{50} values of each regimen were calculated (Table 1). Here, 3 chemotherapeutics (5-FU, PTX and ADR) were used for treatment with or without Bv or Apa. The results showed that both Bv and Apa significantly reduced the IC_{50} value of 5-FU in LoVo cells in a concentration-dependent manner. Moreover, the enhanced cytotoxicity of 5-FU induced by Bv and Apa could be counteracted by tipiracil hydrochloride (TH), the inhibitor of TP. However, neither Bv nor Apa enhanced cell sensitivity of PTX or ADR, as we could see from the almost unchanged IC_{50} values with or without Bv and Apa.

3.7 | Thymidine phosphorylase upregulation induced by bevacizumab depended on vascular endothelial growth factor A/receptor vascular endothelial growth factor receptor 2 pathway and specificity protein 1 phosphorylation

As Bv increased TP expression at both transcriptional and translational level, it was assumed that Bv might affect the transcription

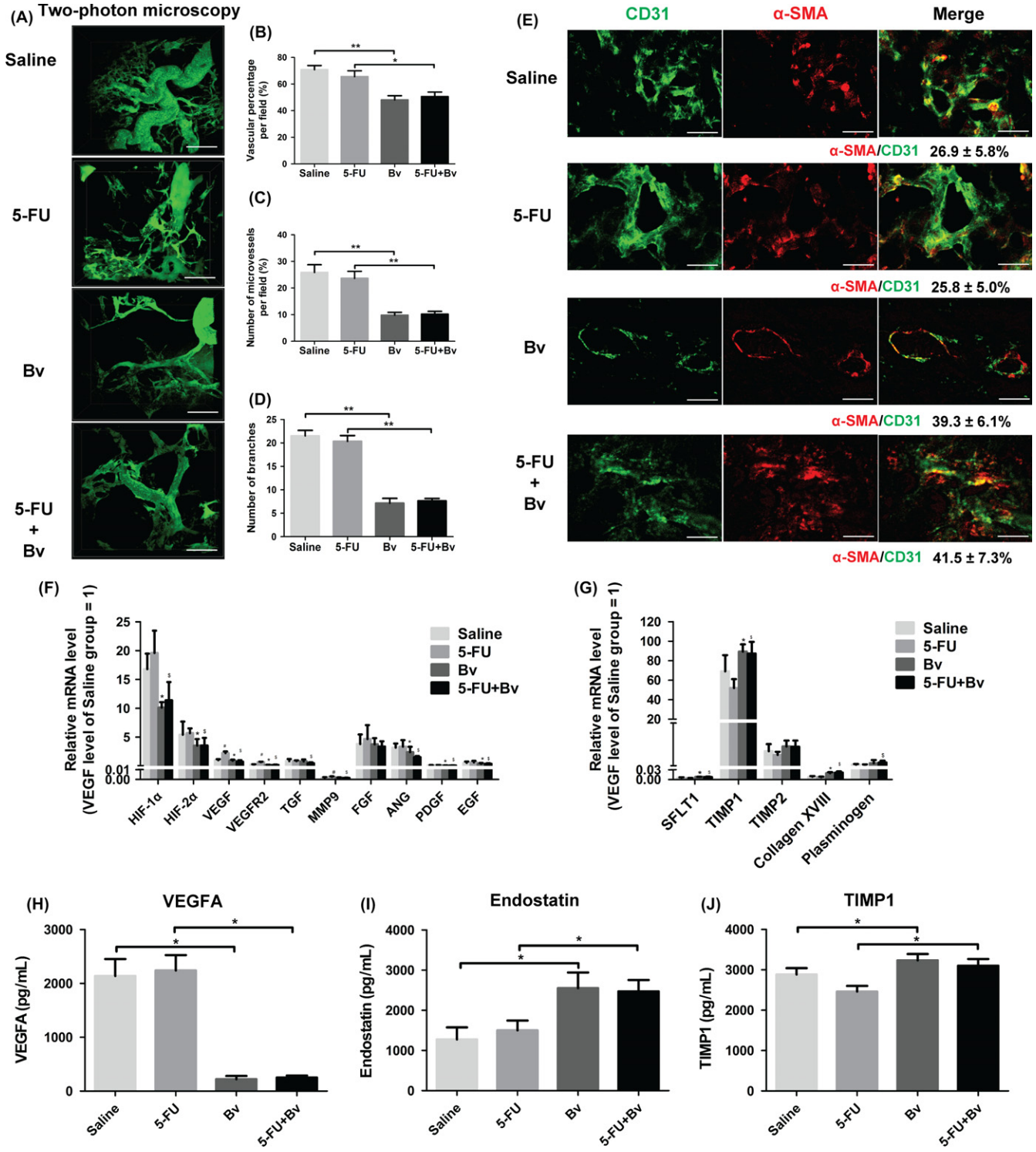

FIGURE 4 Effects of bevacizumab (Bv) on tumor vasculature. A, Tumor angiogenesis imaged with in vivo multiphoton microscopy. Images represent 3-D reconstructions (600 × 600 × 300 mm) acquired by MPLSM. Scale bar = 100 μm, n = 6. B-D, The quantification of vascular volume, number of microvessels and number of branches using ImageJ software. n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$. E, Tumor vessels were immunostained for CD31 (FITC-conjugated, green) and pericytes for α-SMA (Alexa Fluor 680-conjugated secondary antibody, red). 400 ×, scale bar = 30 μm, n = 6. F, Q-PCR assay for tumor proangiogenic factors, n = 8. (G) Q-PCR assay for tumor antiangiogenic factors, n = 8. $^{*}P < 0.05$ between Bv vs saline group; $^{\#}P < 0.05$ between 5-fluorouracil (5-FU) vs saline group; $^{\$}P < 0.05$ between Bv plus 5-FU group vs 5-FU group. H-J, ELISA for VEGFA, endostatin and TIMP1 secretion in tumor tissues, n = 8, $^{*}P < 0.05$

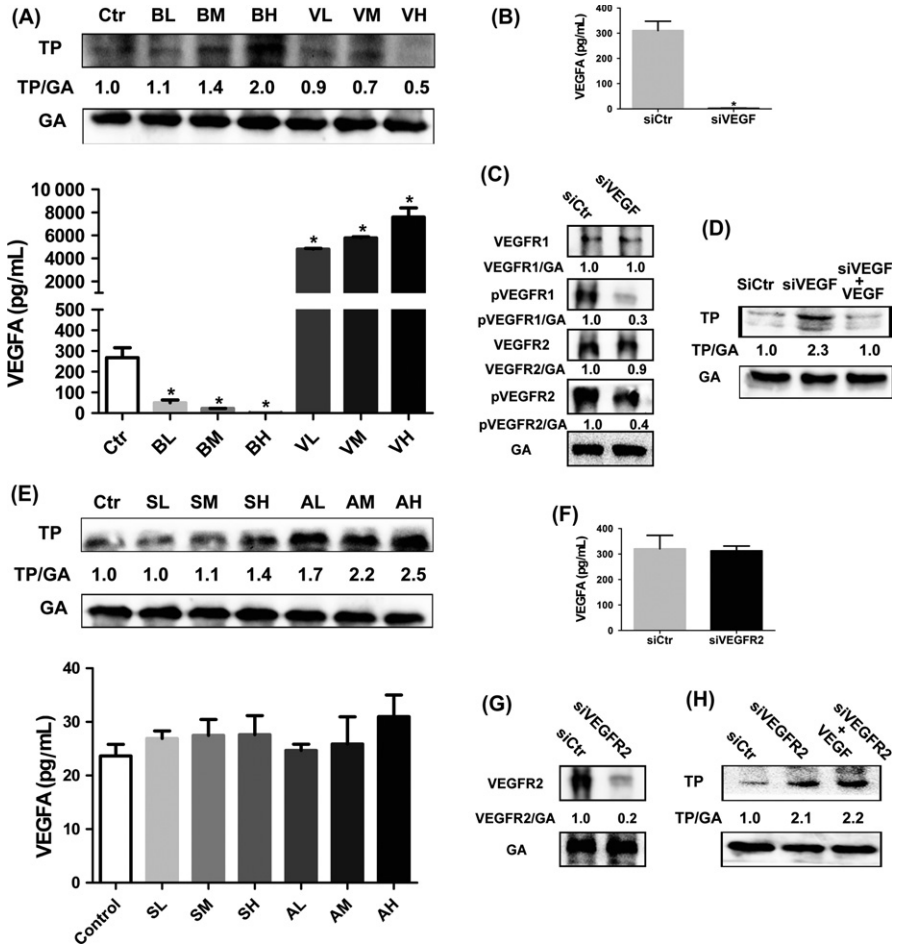

FIGURE 5 Effects of bevacizumab (Bv) and the VEGFR pathway on TP expression in LoVo cells. A, Effects of Bv on TP expression. BL, BM and BH represent 1, 3 and 10 μg/mL bevacizumab, respectively; VL, VM and VH represent 3, 10, 30 ng/mL VEGFA, respectively. B, The efficacy of VEGFA silence detected by ELISA assay. n = 6. C, Effects of VEGFA silence on VEGFR1 and VEGFR2 expression and phosphorylation. D, Effects of VEGFA silencing on TP expression. siCtr represents NC siRNA; siVEGF represents VEGFA silencing; siVEGF + VEGF represents 30 ng/mL; VEGFA added after VEGFA silencing. E, Effects of VEGFR1 or VEGFR2 antagonist on TP expression. SL, SM and SH represent 3, 10 and 30 nmol/L sunitinib (VEGFR1 antagonist), respectively; AL, AM and AH represent 3, 10 and 30 nmol/L apatinib (VEGFR2 antagonist), respectively. F, The efficacy of VEGFR2 silencing. D, Effects of VEGFR2 silencing on TP expression. siVEGFR2 represents VEGFR2 silencing; siVEGFR2 + VEGF represents 30 ng/mL; VEGFA was added after VEGFR2 silencing. Western blots for TP protein in LoVo cells, n = 6. ELISA for VEGFA secretion in LoVo cell medium, n = 6. *$P < 0.05$ between treatment vs control groups

factor specificity protein 1 (Sp1) of TP. First, the effect of mithramycin (inhibitor of Sp1) on Bv-induced expression of TP was evaluated. As shown in Figure 6A, Bv (3 μg/mL) hardly influenced Sp1 expression but significantly induced Sp1 phosphorylation, the activated form of Sp1. Meanwhile, mithramycin blocked the upregulation of TP induced by Bv in LoVo cells in a concentration-dependent manner. Similarly, TP upregulation induced by apatinib (10 nmol/L) was also retarded by mithramycin (Figure 6B). This indicated that the upregulation of TP by Bv might depend on VEGFA/VEGFR2 pathway blockade and Sp1 phosphorylation. Figure 6C,D shows that the silence of VEGFA or VEGFR2 induced Sp1 phosphorylation without change in the total Sp1 level. When adding recombinant VEGFA artificially, upregulation of TP was reversed in the VEGFA silence group but not in the VEGFR2 silence group, reflecting the integrality of VEGFA and VEGFR2 in the modulation of TP expression.

3.8 | The activation of Sp1 by bevacizumab was related to murine double minute-2

It has been reported that the activation of the VEGF/VEGFR pathway could stimulate the expression and function of murine double minute-2 (MDM2), which would induce degradation of Sp1. Therefore, we hypothesized that the inhibition of the VEGF/VEGFR2 pathway might suppress MDM2, leading to the enhancement of Sp1 expression or activation. First, we detected the effect of VEGF/VEGFR activation or inhibition on MDM2 expression. As shown in Figure 7A, when blocking VEGF/VEGFR pathway by Bv, MDM2 was significantly downregulated; when stimulating this pathway by recombinant VEGFA, MDM2 was upregulated. In addition, the silence of VEGFA or VEGFR2 both inhibited MDM2 expression significantly and the re-supplement of VEGFA could restore the MDM2 level in the VEGFA silence group but could not in the VEGFR2 group (Figure 7B). Then MDM2 was silenced to explore its influence on the Sp1/pSp1 level. Results showed that MDM2 silence induced Sp1 phosphorylation without changing the Sp1 level (Figure 7C), which was similar to the effect of Bv. This data indicated that Bv's effect on Sp1 activation was closely related to MDM2.

4 | DISCUSSION

We studied how Bv increases the efficacy of 5-FU in a LoVo colon cancer xenograft mouse model and noted that Bv exerted little effect on 5-FU metabolism in the liver but enhanced the concentration of 5-FU in tumors. Bv upregulated TP in LoVo cancer tissues and cells, and TP played a key role in the metabolic activation of 5-FU chemotherapy rather than promoting angiogenesis as PD-ECGF.

TABLE 1 Effects of bevacizumab (Bv) and Apa on the IC_{50} values of chemotherapeutic drugs

Chemicals	IC_{50} (μmol/L)	Chemicals	IC_{50} (μmol/L)
5-FU	36.45 ± 9.09		
+Bv-L (3 μg/mL)	23.34 ± 5.12	+Apa-L (10 nmol/L)	24.43 ± 4.96
+Bv-H (10 μg/mL)	7.47 ± 2.31	+ Apa-H (30 nmol/L)	8.94 ± 2.43
+Bv-H + TH (100 nmol/L)	28.15 ± 7.11	+Apa-H+TH (100 nmol/L)	30.52 ± 3.56
PTX	0.114 ± 0.223		
+Bv-L (3 μg/mL)	0.104 ± 0.251	+Apa-L (10 nmol/L)	0.1051 ± 0.261
+Bv-H (10 μg/mL)	0.100 ± 0.203	+Apa-H (30 nmol/L)	0.103 ± 0.252
ADR	0.844 ± 0.221		
+Bv-L (3 μg/mL)	0.783 ± 0.20	+Apa-L (10 nmol/L)	0.786 ± 0.258
+Bv-H (10 μg/mL)	0.751 ± 0.310	+Apa-H (30 nmol/L)	0.761 ± 0.281

Data are means ± SEM, n = 5. LoVo cells treated with 5-FU (0.3, 1, 3, 10, 30, 100 μmol/L), PTX (1, 3, 10, 30, 100, 300 nmol/L) or ADR (0.01, 0.03, 0.1, 0.3, 1, 3 μmol/L) alone or with Bv-L (3 μg/mL), Bv-H (10 μg/mL), Apa-L (10 nmol/L) or Apa-H (30 nmol/L). The concentration of TH combined with 5-FU was 100 nmol/L.

TP upregulation by Bv was dependent on blockade of the VEGFA/VEGFR2 pathway and downstream Sp1 activation.

It is worth noting that the most researchers attribute Bv's chemosensitization effect to its impact on tumor microvessels. Bv could exert anti-angiogenesis and block the blood and nutrition supply in tumors,[18] and some papers have revealed that Bv can promote tumor vascular normalization to promote blood perfusion and drug delivery into tumor tissues.[19,20] However, the literature contains few studies on the effects of Bv on 5-FU metabolic enzymes. In the present study, Bv did, as expected, enhance the antitumor effect of 5-FU in a LoVo colon cancer xenograft model, accompanied by the increased 5-FU concentration in tumors (Figures 1 and 2). Whether these changes were due to 5-FU metabolism was not clear.

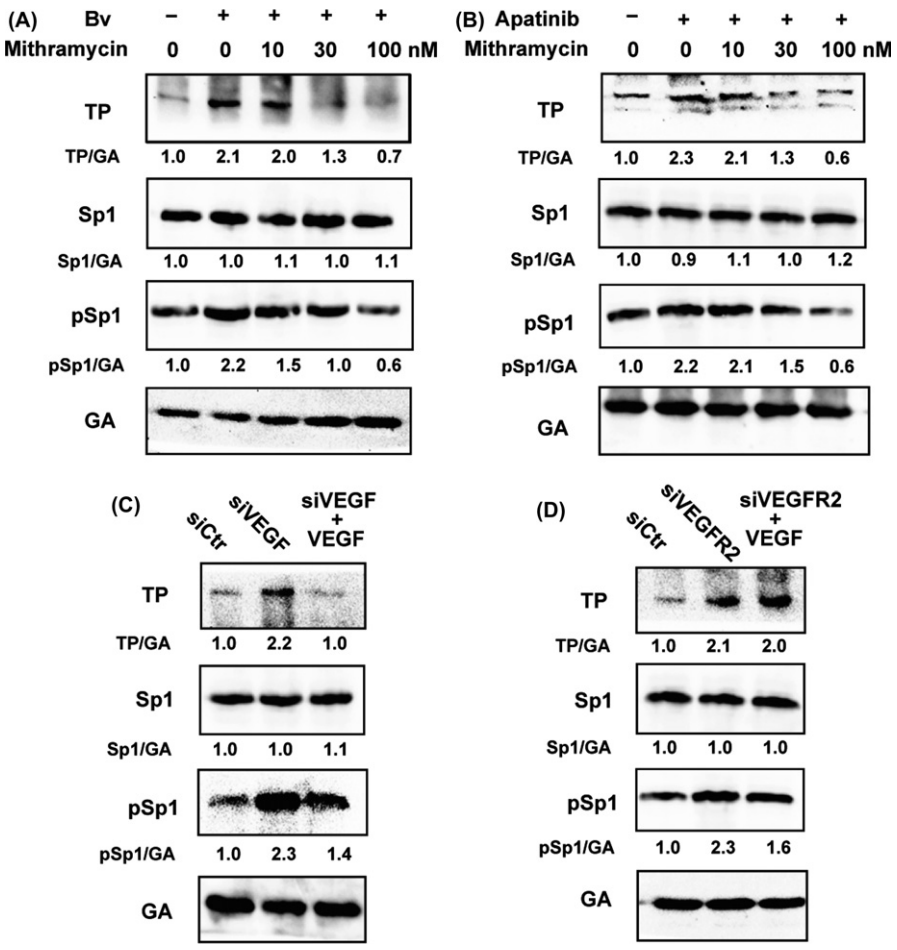

FIGURE 6 Effects of bevacizumab (Bv) and the VEGFR2 pathway on TP expression and Sp1 activation in LoVo cells. A, Effects of Bv and Sp1 inhibitor mithramycin on TP expression and Sp1 phosphorylation. TP, Sp1 and phosphorylated-Sp1 (pSp1) protein were measured after 24 h treatment with Bv (3 μg/mL) combined with different concentrations of mithramycin. B, Effects of apatinib and mithramycin on TP expression and Sp1 phosphorylation. Cells were treated for 24 h with apatinib (10 nmol/L) combined with different concentrations of mithramycin. C, Effects of VEGFA silencing on TP expression and Sp1 phosphorylation. siVEGF represents VEGFA silencing; siVEGF + VEGF represents 30 ng/mL; VEGFA was added after VEGFA silencing. D, Effects of VEGFR2 silencing on TP expression and Sp1 phosphorylation. siVEGFR2 represents VEGFR2 silencing; siVEGFR2 + VEGF represents 30 ng/mL; VEGFA was added after VEGFR2 silencing. TP, Sp1 and pSp1 protein were measured. n = 6

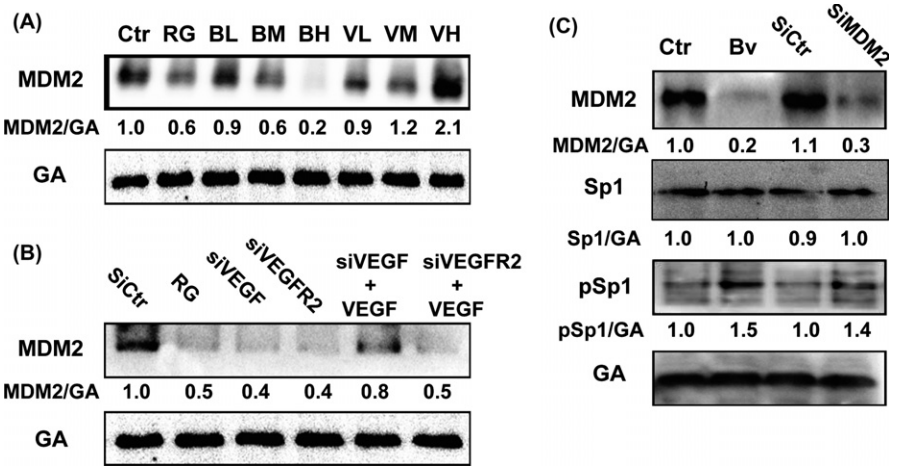

FIGURE 7 Regulatory mechanism of Sp1 activation induced by bevacizumab. A, Effects of VEGF/VEGFR pathway inhibition or activation on MDM2 expression. RG represents 30 nmol/L RG7112, the inhibitor of MDM2; BL, BM and BH represent 1, 3 and 10 μg/mL bevacizumab, respectively; VL, VM and VH represent 3, 10 and 30 ng/mL VEGFA, respectively. B, Effects of VEGFA or VEGFR2 silence on MDM2 expression. RG represents 30 nM RG7112. C, Effects of MDM2 silence on Sp1 phosphorylation. Bv represents 10 μg/mL bevacizumab. n = 6

Drug metabolism is an important procedure closely related to therapeutic effects and multidrug resistance.[21] We found that Bv had little effect on 5-FU concentration in plasma and liver (Figure 2A,B) as well as on the concentration of hepatic inactivated metabolite 5-FUH_2 (Figure 2C). These data were further confirmed by hepatic S9 protein incubation assays (Figure 2E,F) and liver DPD expression data (Figure 2G,H). DPD, mainly expressed in the liver, metabolizes more than 80% of 5-FU, and its deficiency or overexpression can cause toxicity or resistance for patients treated with fluoropyrimidine drugs, such as 5-FU or capecitabine.[22] Our data suggested that Bv plus 5-FU does not cause hepatic drug-drug interactions. Similarly, no significant pharmacokinetic interactions were observed when Bv was given with capecitabine in advanced CRC patients.[23] However, whether Bv enhanced 5-FU in tumors through regulating enzymes mediating 5-FU activation to inhibit DNA or RNA synthesis in cancer was studied.

In cancer cells, TP converts 5-FU to FdUrd, which is then phosphorylated by TK to 5-FdUMP. 5-FdUMP directly binds to TS and inhibits DNA synthesis. On the other side, 5-FU could be transformed to FUMP by UP, UK and OPRT to induce RNA damage. In the present study, TP presented the highest amount of expression and the most obvious change among these 3 enzymes after Bv treatment (Figure 3). In addition to its metabolic function, TP, which is identical to PD-ECGF, has various biological functions, such as promoting tumor angiogenesis, invasion, metastasis and resistance to apoptosis.[24,25] In the present study, is the result of the elevated TP expression induced by Bv good or bad for chemotherapy? According to the comprehensive data, including on anti-angiogenesis and vascular normalization (Figure 4), it can be inferred that in our study the elevated TP by Bv tends to increase the sensitivity of cancer cells to 5-FU rather than promote tumor angiogenesis. Although TP can increase the production of VEGFA, the combined administration of Bv neutralizes VEGFA and blocks its binding to VEGFR to inhibit angiogenesis signals. However, TP can also protect cancer cells from apoptosis induced by cisplatin or antimicrotubular drugs,[26-28] which is independent of the VEGFA/VEGFR pathway. In vitro, we noted that Bv and Apa significantly enhanced the cytotoxic effects of 5-FU but could not strengthen the cytotoxic effects of PTX and ADR (Table 1). 5-FU may prefer to be converted to its activated form by TP upregulation, resulting in enhancing cytotoxicity in cancer cells. However, PTX and ADR did not need TP for activation, resulting in TP accumulation, which may cause drug resistance and poor prognosis. Therefore, Bv used with ADR, antimicrotubular drugs or cisplatin may accelerate resistance to chemotherapy by upregulating TP.

Accumulating evidence suggests that increased VEGFA exists in high TP-expressing tumors.[29,30] However, few papers have focused

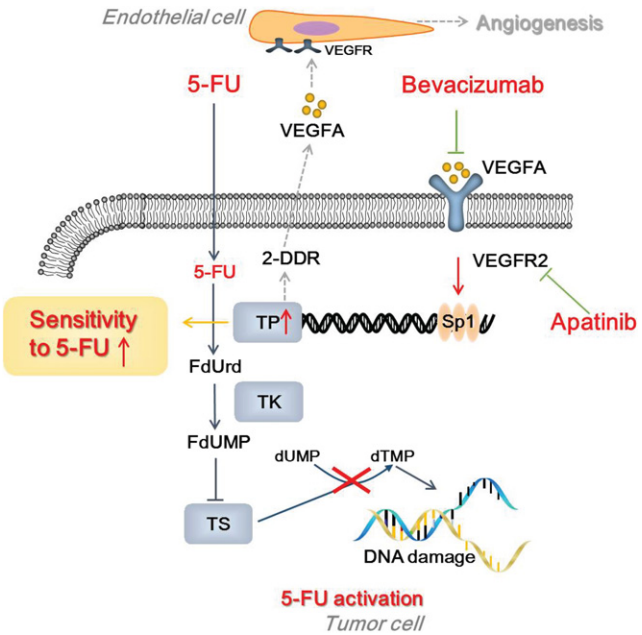

FIGURE 8 A schematic diagram elucidating the potential mechanism underlying the synergistic effect of bevacizumab (Bv) on 5-FU. Bv increased TP expression, which could facilitate VEGFA secretion and promote tumor angiogenesis. However, in tumor cells, elevated TP by Bv can drive 5-FU metabolic activation forward and damage DNA synthesis. In contrast, the promotion of angiogenesis or resistance by TP may be diminished. This suggests the superiority of Bv in combination with fluoropyrimidine drugs compared to other chemotherapeutic drugs

on the effect of the VEGFA/VEGFR pathway on TP expression. We found that TP upregulation by Bv depended on the VEGFA/VEGFR2 pathway rather than the VEGFA/VEGFR1 pathway (Figure 5). It was reported that the promoter of either TP or VEGFA contained multiple Sp1 transcription factor-binding sites. TP and VEGFA are similarly regulated by Sp1 binding sites.[31,32] Therefore, it is understandable that VEGFA and TP may be co-expressed in some human cancers. However, TP is also reported to be an independent prognostic factor compared to VEGFA.[33] This suggests that TP and VEGFA expression are not always required to be coordinated. It has been reported that astrocytic TP induction by IL-1β in vivo was potentiated by conditional VEGFA inactivation, but the underlying mechanism was not studied.[34] We assumed that VEGFA diminished by Bv might enhance TP expression in the feedback loop based on Sp1 activation, which was confirmed by the data in Figure 6. This indicates that long-term treatment with VEGFA/VEGFR2 inhibitors such as apatinib and vandetanib leads to the risk of inducing TP overexpression, promoting cancer progression or resistance. Finally, we preliminarily explored how the Sp1 was activated by VEGF/VEGFR2 block. Studies have reported that overexpression of MDM2 would induce Sp1 degradation, and VEGF/VEGFR pathway activation might promote MDM2 expression and function. Therefore, we assumed that the inhibition effect on the VEGF/VEGFR2 pathway of Bv might suppress MDM2 expression, resulting in the enhancement of Sp1 activation. This hypothesis is confirmed, as shown in Figure 7.

Thus, we proposed that Bv enhanced tumor delivery and cytotoxicity of 5-FU in CRC via tumor vessel normalization and TP upregulation, and how this may occur is shown in Figure 8. Multiple treatments with Bv can elevate TP expression via the blockade of the VEGFA/VEGFR2 pathway and Sp1 activation, which may lead to a risk of promoting angiogenesis or resistance. However, when Bv is co-administrated with 5-FU, upregulated TP may intensify 5-FU conversion to its active metabolites and its subsequent cytotoxicity to cancer cells. Then, the promotion of angiogenesis or resistance by TP may be diminished. We offer data to better understand how Bv synergizes with 5-FU from a metabolic perspective, and this may direct future therapeutic approaches for chemotherapy.

DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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