Targeting tumor cell-derived CCL2 as a strategy to overcome bevacizumab resistance in ETV5+ colorectal cancer

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Research

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Abstract

Background

ETV5 mediated angiogenesis was dependent on PDGF-BB/PDGFR-β/Src/STAT3/VEGFA pathway in colorectal cancer (CRC) in our previous study. However, whether ETV5 affects effect of antiangiogenic therapy in CRC requires further investigation.

Methods

GSEA analysis and a series of experiments were performed to identify the critical candidate gene involved in bevacizumab resistance, and further explored whether the treatment targeting the candidate gene enhanced bevacizumab sensitivity in vitro and in vivo.

Results

ETV5 could directly bind to VEGFA promoter and promote translation of VEGFA. However, by *in vitro* and *in vivo* experiments, ETV5 actually accelerated anti-VEGF therapy (bevacizumab) resistance. GSEA analysis and further assays confirmed that ETV5 could promote angiogenesis by enhancing secretion of another tumor angiogenesis factor CCL2 in CRC cells, which resulted in bevacizumab resistance. ETV5 induced VEGFA and CCL2 were mutually independent to induce angiogenesis by activating PI3K/AKT and p38/MAPK signaling pathways in human umbilical vein endothelial cells. In CRC tissues, ETV5 protein level was positively associated with CD31, CCL2, VEGFA protein expression. CRC patients with high expression of ETV5/VEGFA or ETV5/CCL2 showed inferior prognosis than other patients. Combination of anti-CCL2 and anti-VEGFA (Bevacizumab) treatment could more effectively inhibited tumor angiogenesis and growth than single treatment did in CRCs with high expression of ETV5 (ETV5 + CRCs).

Conclusions

Our results not only revealed ETV5 as a novel biomarker for antiangiogenic therapy, but also indicated a potential combined therapy strategy by simultaneously targeting CCL2 and VEGFA in ETV5 + CRC.

Background

Colorectal cancer (CRC) is one of the most common cancers worldwide, with morbidity and mortality ranking third among cancers[1]. Despite advancements in the diagnosis and treatment of CRC in the past few decades, the prognosis still remains poor [2]. Angiogenesis is a hallmark process in the oncogenesis of CRC [3–5], and vascular endothelial growth factor A (VEGFA) and its receptors (VEGFR-1/-2) play dominant roles in this complex regulation. Attenuating VEGF-VEGFR signaling could disrupt vascularization and has been considered to be a promising therapeutic strategy for CRC [6].

Bevacizumab, a clinically used antiangiogenic drug, can specifically target VEGFA and then inhibit VEGF-VEGFR signaling [6]. Bevacizumab plus chemotherapy is the first-line treatment option for metastatic CRC [6–8]. Nonetheless, some CRC patients are resistant to Bevacizumab, and the overall response rate is limited [9]. To further explore the angiogenesic mechanisms underlying the antiangiogenesic resistance is conducive to enhancing bevacizumab sensitivity in CRC.

The ETS transcription factor family contains 28 factors and can be divided into 12 subfamilies [10]. E26 transformation-specific variant 5 (ETV5), a member of the ETS family, has been reported to be involved in the progression of hematologic malignancies, endometrial cancer [11, 12], ovarian cancer [13, 14], prostate cancer and thyroid cancer [15, 16]. Some previous studies also revealed that the ETS family triggered angiogenesis in multiple tumors [13, 17, 18]. Similarly, our previous study showed that ETV5 facilitated CRC angiogenesis via PDGF-BB/Src/STAT3/VEGFA signaling pathway [19]. Besides, more and more studies indicated that chemokine signaling played critical roles in tumor angiogenesis, such as CCL2/CCR2 signaling and CXCL11/CXCR7 signaling [20, 21]. ETV5 was also reported to regulate chemokines expression of sertoli cell in mouse, such as Ccl7, Ccl9, Ccl12. The mechanism of paracrine chemokine signaling are still a critical drug resistance in cancers [22]. However, how ETV5 regulates anti-VEGF therapy sensitivity and underlying mechanisms require further clarification.

In the present study, we found that ETV5 could promote bevacizumab resistance via secretion of CCL2, and CCL2 could also induce angiogenesis by activating PI3K/AKT and p38/MAPK signaling pathways in umbilical vein endothelial cells. Combination inhibition of bevacizumab and CCR2 inhibitor showed synergistically inhibitory effect on tumor growth. Our results indicated that an approach simultaneously targeting CCL2 and VEGFA in ETV5 + CRC.

Materials And Methods

Patient specimens

The patient information has been described previously [23]. In brief, tissues and adjacent nontumorous tissues were collected following a protocol from 75 CRC patients who underwent surgery at Ruijin Hospital from 2010 to 2011. We recorded clinical parameters, pathological data, overall survival (OS), and disease-free survival (DFS).

Cell Lines, Bevacizumab And Chemical

Human CRC cell lines RKO and HT29 cells were purchased from American Type Culture Collection (ATCC). HUVECs were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) and antibiotics at 37 °C with 5% CO2. Bevacizumab (a humanized monoclonal antibody that specifically binds to all VEGFA isoforms with high affinity) was purchased from MedChemExpress (Cat. No. HY-P9906). Anti-CCL2 (clone

2H5) antibody, an IgG monoclonal antibody neutralizing the bioactivity of human natural or recombinant CCL2, was purchased from BioLegend (Cat. No. 505913).

RNA Extraction And Quantitative RT-PCR

Total RNA was extracted by TRIzol (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Invitrogen, CA). Quantitative PCR was performed using TaqMan® Gene Expression Assays (Thermo Fisher Scientific). The premiers were listed as follows: CXCL11: (forward) 5'-GACGCTGTCTTTGCATAGGC-3', (reverse) 5'-GGATTTAGGCATCGTTGTCCTTT-3'; CXCL5, (forward) 5'-GAGAGCTGCGTTGCGTTTGTTTAC-3', (reverse) 5'-CCGTTCTTCAGGGAGGCTACCACT-3'; CCL2, (forward) 5'-CAGCCAGATGCAATCAATGCC-3', (reverse) 5'- TGGAATCCTGAACCCACTTCT – 3'; CCL13, (forward) 5'- TGCTGACCCAAAGGAGAAG-3', (reverse) 5'- GCCAGAGGAGAATGGAAAAG-3'.

Western blot analysis

Western blotting was performed as previously described [23]. Briefly, cells were harvested at various time intervals and lysed in RIPA buffer in the presence of a Protease Inhibitor Cocktail (Pierce, USA) and a Protein Phosphatase Inhibitor Cocktail (New Cell & Molecular Biotech, China). One hundred micrograms of protein was separated by 10% SDS-PAGE gel and transferred to PVDF membranes (Tanon, China). The membranes were blocked with 5% bovine serum albumin (BSA) for 2 h and then incubated overnight with primary antibodies at 4 °C. The blots were probed with anti-pP38 (4511S, 1:1000, Boston, USA), anti-P38 (9212S, 1:1000, Boston, USA), anti-AKT (4691S, 1:1000, Boston, USA), anti-pAKT (4060S, 1:1000, Boston, USA), and anti-GAPDH (ab8245, 1:10000, Cambridge, UK); GAPDH was used as the internal control. Goat anti-mouse or goat anti-rabbit horseradish-peroxidase-conjugated IgG was used as the secondary antibody (Santa Cruz Biotechnology). The membranes were incubated with secondary antibody for 2 h at room temperature, and bands were visualized using an enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ, USA) according to the manufacturer's protocol.

Generation Of Gene Overexpressing And Knocked Down Cells

Lentiviruses for ETV5 and VEGFA overexpression and knockdown were purchased from Shanghai Bioegene Co., Ltd. (Shanghai, China). Lentiviral particles were transduced into CRC cells according to the manufacturer's instructions, followed by stable selection. Lentivirus transfection was performed as described previously [19]. Antibiotic puromycin was used to select stably transfected cells. The effects of overexpression and knockdown were evaluated by western blotting.

Cell Viability And Colony Formation Assays

Cell viability and colony formation assays were performed as described previously [19]. Approximately 3000 cells were plated in 96-well plates and cultured in a 37 °C/5% CO2 incubator. Cell viability was detected in a time series with CCK-8 (Dojindo Molecular Technologies Inc.). For the colony formation assay, 1,000 cells were plated per well in 6-well plates and cultured at 37 °C for 2 weeks. Colony formation was calculated by staining with 0.1% crystal violet in methanol for 30 min.

Endothelial Tube Formation Analysis

Human umbilical vein endothelial cells (HUVECs) were treated with supernatants of CRC cell lines in 96-well plates at a density of 1×10^4 cells per well at 37 °C. Each plate was precoated with 50 μL Matrigel (BD Bioscience) at 37 °C for 30 min. After a 6-h incubation, tubules were observed by microscopy and analyzed using Image-Pro Plus software.

Chick Embryo Chorioallantoic Membrane (CAM) Assay

The CAM assay was performed as previously described [3]. Filter paper was placed on eggs, with a round window cut in the egg shell in advance, and then 30 μL of the cell culture supernatant was dropped onto the filter paper tray, which was sealed with a transparent tape for 3 days. On the 10th day, the eggs were imaged using a MacroPATH dissecting microscope (Milestone, Italy), and the number of blood vessels around the filter paper tray was counted.

In vivo **PC xenograft tumor model**

The CRC xenograft mouse model was performed as previously described, and all mice were euthanized one month after injection [23]. Tumors were weighed and fixed in formalin. All animal studies were approved by the Ruijin Hospital's Ethics Committee, Shanghai Jiao Tong University School of Medicine.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed following the chromatin immunoprecipitation kit (Millipore) protocol, as previously described [19]. Briefly, an anti-ETV5 antibody (Santa Cruz Biotechnology, SC-22807) was used for immunoprecipitation. After purification of the precipitated DNA, the human VEGFA promoter was amplified by qRT-PCR. The premiers used to amplify the two site of VEGFA promoters were listed as: forward, 5’-TAGTGCTGGCGGGTAGGTTT-3’; reverse, 5’-CCAAGTTTGTGGAGCTGAGAA-3’.

ELISA

ELISA was performed according to previously described methods [24, 25]. In brief, CRC cell lines were seeded in six-well plates and incubated for several days. The VEGFA and CCL2 expression in supernatant

was detected by using the VEGFA Human Biotrak ELISA system (Amersham Biosciences Corp., Piscataway, NJ) and Human MCP1 (CCL2) ELISA kit (ab179886, Abcam, Cambridge, UK).

Luciferase Report Assay

ETV5 promoter fragments were amplified from human genomic DNA and cloned into the pGL3-Basic vector. Luciferase activity was examined using the Dual-Luciferase Assay (Promega) following the manufacturer's instructions.

Immunohistochemistry Assay

FFPE slides with CRC specimens and nude mice tumor tissue sections were stained as previously described [3, 19]. Antibodies used for IHC included antibodies against ETV5 (ab102010, Abcam), VEGFA (ab46154, Abcam), CD31 (3528, Cell Signaling Technology), Ki67 (1:200, Santa Cruz), and CCL2 (ab9669).

GSEA Analysis

The transcription data of raw count of 635 CRCs were downloaded from TCGA (The Cancer Genome Atlas) database (https://portal.gdc.cancer.gov/). Then, the transcripts per million (TPM) of every gene was calculated and normalized by Log2(TPM + 1). According to the median of ETV5 gene expression value, we divided all CRCs into High (n = 212), Moderate (n = 211) and Low (n = 212) groups. By Gene Set Enrichment Analysis (GSEA) software (https://www.gsea-msigdb.org/gsea/index.jsp), the enriched KEGG pathways were analyzed between High and Low groups.

Statistics

The genes significantly enriched in Chemokine Signaling Pathway were displayed by heatmap using "pheatmap" package in R software. The gene expression value of CXCL11, CXCL5, CCL2 and CCL13 were extracted from our previous RNA-Seq data of HT29/Vector and HT29/shETV5 cells, which had been deposited in GEO database (GSE112628). Data are expressed as the means ± SD. Analysis of variance (ANOVA) and Student's t-test were employed for comparisons among groups. The Mann-Whitney U-test was applied for the tumor volume comparison. Categorical data were evaluated with the chi-square test or Fisher's exact test. ROC curve was plotted to determine the cutoff values for ETV5, VEGFA and CCL2 expression. Log-rank test was performed to compare the survival curves of two or more groups. P-values less than 0.05 were considered significant. Statistical analyses were processed using

Results

ETV5 induces bevacizumab resistance in CRC

Our previous study showed that ETV5 significantly promoted CRC angiogenesis *in vitro* and *in vivo* [19], but the underlying mechanisms still need to be clarified. Furthermore, we analyzed the promoter sequence of the VEGFA gene and detected a potential ETV5 binding site using jaspr software (Figure S1a). Chromatin immunoprecipitation (ChIP) assays for anti-ETV5 were performed in RKO cells, followed by qRT-PCR of the VEGFA promoter and upstream regions, and the results revealed that the ETV5 protein could bind to the site in VEGFA promoter (Figure S1b). Moreover, the results from a luciferase reporter assay showed less luciferase expression for VEGFA-promoter-ETV5 compared to VEGFA-promoter-Vector (Figure S1c) and that ETV5 was able to activate the wild type VEGFA promoter but not the mutant promoter (Figure S1d).

Our previous study found that PDGF-BB could activate VEGFA expression via the PDGFR-β/Src/STAT3 pathway in CRC [19], and simultaneously the present results indicated ETV5 could upregulate VEGFA via transcriptional activation of VEGFA in CRC. ETV5 knockdown attenuated angiogenesis could be reversed by recombinant human VEGFA (Fig. 1a). In cancers, high expression of VEGFA was particularly related to sensitivity of VEGFA inhibition, including liver cancer, sarcoma and breast cancer [26–28]. However, by chick embryo chorioallantoic membrane (CAM) assay, ETV5 upregulation in RKO promoted bevacizumab resistance (Fig. 1a). In vivo, after receiving bevacizumab treatment, RKO/ETV5 tumors grew faster than RKO/Vector tumors (Fig. 1b). Four weeks later, the mice were sacrificed, and the subcutaneous tumors were removed for measure. The size of tumors in RKO/ETV5 + Bev group were larger than tumors in RKO/Vector + Bev group (Fig. 1c and Fig. 1d). In the tumor tissues, RKO/ETV5 + Bev group showed higher expression of VEGFA and CD31 than RKO/Vector + Bev group (Fig. 1e).

Based on CCK8 and two-dimensional colony formation assays, similar results were also observed. ETV5 knockdown attenuated cell proliferation and tubular formation of human umbilical vein endothelial cells (HUVECs) could be reversed by recombinant human VEGFA protein (Fig. 2a and Fig. 2b). After receiving bevacizumab treatment, RKO/ETV5 group showed more apparent cell proliferation and tubular formation of HUVECs than RKO/Vector group (Fig. 2a and Fig. 2b).

CCL2 is identified as another critical proangiogenic factor regulated by ETV5 in CRC.

The above results indicated that ETV5 upregulation leaded to bevacizumab resistance. In order to explore the potential mechanisms. We use TCGA data to perform ETV5-related GSEA analysis. According to the ETV5 expression, we firstly divided all CRCs into three groups: High (n = 212), Moderate (n = 211) and Low (n = 212) groups. GSEA analysis were performed between High group and Low group. It was worth noting that ETV5 positively related to activation of chemokine signaling pathway (Fig. 3a), which was involved in angiogenesis in cancers [29, 30]. In this pathway, 26 genes were significantly enriched and further displayed by heatmap (Fig. 3b). Among them, four genes encoding proangiogenic proteins, such as CXCL5, CXCL11, CCL2 and CCL13. In our previous dataset (GSE112628) deposited in GEO database, only expression of CCL2 was higher HT29/Vector than HT29/shETV5 cells (Fig. 3c), which was further

validated by qRT-PCR (Fig. 3d). By ELISA, higher level of CCL2 was examined in culture supernatants of HT29/Control than HT29/shETV5cells, and higher level of CCL2 was detected in culture supernatants of RKO/ETV5 than HT29/Vector cells (Fig. 3e). In vivo, we also found that RKO/ETV5 + Bev group showed higher expression of CCL2 than RKO/Vector + Bev group (Fig. 3f).

CCL2 Partly Contributes ETV5-mediated Angiogenesis In CRC

We found that ETV5 downregulation suppressed CCL2 secretion was not rescued by recombinant human VEGFA, and ETV5 upregulation induced CCL2 secretion was also not reversed by bevacizumab in HT29 (Fig. 4a). ETV5 downregulation suppressed VEGFA secretion was not rescued by recombinant human CCL2 protein, and ETV5 upregulation induced CCL2 secretion was also not reversed by bevacizumab in RKO (Fig. 4a). By two-dimensional colony formation and CAM assays, ETV5 upregulation induced tubular formation of HUVECs could be reversed by anti-CCL2 antibody, and ETV5 knockdown attenuated tubular formation of HUVECs could be rescued by recombinant human CCL2 protein (Fig. 4b and Fig. 4c). These results indicated that ETV5 promoted angiogenesis by enhanced CCL2 secretion, which was independent of VEGFA.

Combination of anti-VEGFA and anti-CCL2 showed synergistic anti-tumor effect in CRC.

The above results revealed two secreted proangiogenic proteins mediated by ETV5, which accelerated angiogenesis in CRCs with high expression of ETV5 (ETV5 + CRCs). ETV5 mediated secretion of CCL2 may be the critical reason for bevacizumab resistance in CRC. By two-dimensional colony formation, combination of recombinant human CCL2 and VEGFA protein more obviously rescued ETV5 knockdown attenuated tubular formation of HUVECs than single treatment in HT29, and combination of bevacizumab and anti-CCL2 antibody more apparently reversed ETV5 upregulation enhanced tubular formation of HUVECs than single treatment in RKO (Fig. 5a). Meanwhile, the same tendency was observed for AKT and p38 in HUVECs (Fig. 5b). Then, CAM assays were further performed. We found that combination of recombinant human CCL2 and VEGFA protein more obviously inhibit angiogenesis than single treatment in HT29/shETV5 cells, and combination of bevacizumab and anti-CCL2 antibody more apparently suppressed angiogenesis than single treatment in RKO/ETV5 cells (Fig. 5c). In vivo, the tumor in RKO/ETV5 + bevacizumab/anti-CCL2 group grew slower than RKO/ETV5 + bevacizumab or RKO/ETV5 + anti-CCL2 group (Fig. 5d). Four weeks later, the subcutaneous tumors were removed for measure. The size of tumors in RKO/ETV5 + bevacizumab/anti-CCL2 group were also larger than tumors in RKO/ETV5 + bevacizumab or RKO/ETV5 + anti-CCL2 group (Fig. 5e). In the tumor tissues, CD31 and ki67 were higher in RKO/ETV5 + bevacizumab/anti-CCL2 group than that of RKO/ETV5 + bevacizumab or RKO/ETV5 + anti-CCL2 group (Fig. 5f).

ETV5, VEGFA and CCL2 show positive expression correlations with angiogenesis and are positively correlated with poor prognosis in CRC

By IHC staining, we detected ETV5, VEGFA, CCL2 and CD31 protein levels in 75 paired CRC and normal colorectal tissues (Fig. 6a), and found all of them were upregulated in CRC tissues compared with normal tissues (Fig. 6b). We observed a positive correlation between ETV5 expression and VEGFA expression ($r = 0.6089$ $p < 0.0001$, Fig. 6c), CCL2 ($r = 0.2449$ $p = 0.0342$, Fig. 6c) or CD31 ($r = 0.4833$ $p < 0.0001$, Fig. 6c). Furthermore, VEGFA ($r = 0.4370$ $p < 0.0001$, Fig. 6d) and CCL2 ($r = 0.4155$ $p = 0.0002$, Fig. 6d) were also significantly related CD31 expression in CRC tissues. Patients with tumors positive for both ETV5 and VEGFA (Fig. 6e) or both ETV5 and CCL2 exhibited the worst OS and DFS (Fig. 6f). Mechanistically, ETV5 promoted CRC angiogenesis through increased secretion of VEGFA and CCL2. Although receiving bevacizumab treatment, the angiogenesis in ETV5 + CRCs did not be well blocked because another proangiogenic factor CCL2 was also induced by ETV5, which could promote angiogenesis by activating MAPK and AKT pathways in HUVECs. Therefore, additional anti-CCL2 treatment may be a promising method to overcome bevacizumab resistance by strongly inhibiting angiogenesis in CRC (Fig. 7).

Discussion

ETV5 leads to tumor initiation, progression and metastasis by governing many biological processes [31, 32], including cell cycle control, differentiation, proliferation, apoptosis, tissue remodeling and angiogenesis [10]. Molecular stability and upregulation of ETV5 maintains homeostasis and carcinogenesis in breast and prostate cancer [32, 33]. Furthermore, ETV5 has been reported to directly transcript MMP2 and TIMP to modify tumor growth [19, 34]. In CRC, ETV5 promoted angiogenesis through PDGF-BB induced VEGFA in CRC [19]. In the present study, we further revealed that ETV5 directly promoted transcription of proangiogenic factor VEGFA in CRC. Some previous studies reported cancers with high levels of VEGFA were particularly sensitive to VEGFA inhibition, including liver cancer, sarcoma and breast cancer [26–28]. Now that ETV5 could strongly induced VEGA expression in CRC, we speculated that the ETV5 + CRCs should be extremely sensitive to VEGFA. However, when forcing expression of ETV5 in CRC cells, tumors were resistant to anti-VEGF therapy (bevacizumab). Bevacizumab is a molecular-targeted drug that specifically binds to and neutralizes human VEGFA to inhibit VEGF signaling pathway[35]. Although bevacizumab can be used to treat metastatic colorectal cancer [36, 37], bevacizumab resistance limits its therapeutic efficacy. Our results indicated that ETV5 may be a biomarker to assess the bevacizumab resistance in CRC. There may be paracrine activation in the course of medical treatment [22, 38, 39].

In order to explore the potential mechanisms involved in bevacizumab resistance in CRC, we used the TCGA data to performed ETV5-related GSEA analysis. We found that ETV5 expression was significantly associated with activation of chemokine signaling pathway, which could also contribute to angiogenesis in many cancers [29, 30]. In the significantly enriched genes, four genes were also proangiogenic factors, such as CXCL11, CXCL5, CCL2, CCL13 [19]. Among them, only CCL2 was markedly attenuated by ETV5 knockdown in CRC cells. CCL2 was identified as another proangiogenic factor induced by ETV5 in CRC. CCL2/CCR2 chemokine signaling was proved to promote breast cancer progression by induced angiogenesis [20]. By ELISA assay, we found that attenuation of VEGFA could not affect ETV5-mediated CCL2 secretion, and anti-CCL2 treatment also did not have influence on ETV5-mediated VEGFA secretion

in CRC cells. These results hinted that VEGFA and CCL2 may be two parallel signals to induce angiogenesis in ETV5 + CRCs. Further rescue experiments confirmed that ETV5-mediated CCL2 secretion by CRC cells promoted bevacizumab resistance in a manner of paracrine activation effect in HUVECs. Therefore, when the ETV5 + CRCs received bevacizumab treatment, the secreted CCL2 signaling pathway continued to activate angiogenesis-related pathway in HUVECs, such as PI3K/AKT and p38/MAPK signaling pathways, which resulted in angiogenesis [8]. Our resulted confirmed that ETV5-mediated secretion of CCL2 played a crucial role in bevacizumab resistance.

Single drug treatment often leads to resistance in cancers, and combined treatment are necessary [40, 41]. Our results indicated that both VEGFA and CCL2 participated in angiogenesis in ETV5 + CRCs. Besides, the result that CRCs with high expression of ETV5/VEGFA or ETV5/CCL2 showed inferior prognosis than other patients further indicated critical roles of VEGFA and CCL2 in CRC progression. The treatment targeting CCL2 may be a promising method to overcome anti-VEGF therapy resistance of ETV5 + CRCs. In a phase 2 study, carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 (CCL2), showed antitumor activity as a single agent in metastatic castration-resistant prostate cancer [42]. Therefore, we performed a combined treatment of bevacizumab and anti-CCL2. The combined treatment more effectively inhibited tumor growth and angiogenesis than single drug treatment. In HUVECs, phosphorylation of AKT and p38 could promote tubular formation in cancers [43, 44]. Mechanically, in the present study, the combined treatment also more obviously inactivated angiogenesis-related pathway PI3K/AKT and p38/MAPK than single drug did in HUVECs. Due to the complicated signaling pathway and interaction network of multiple proteins, single drug often achieves limited effects in cancers, and more rational combined treatment is acquired [45, 46]. Feig et al reported that the compound AMD3100 targeting CXCL12/CXCR4 signal could overcome anti-CTLA4 and anti-PD1 treatment resistance by depleting carcinoma-associated fibroblasts in pancreatic cancer [47]. Savino et al found that combined targeting of CCR2 and the ERK pathway may be a promising therapeutic strategy in kaposi sarcoma, which involved in inhibition of angiogenesis and tumor growth. Thus, we concluded that the treatment targeting CCL2/CCR2 may be an effective method to reverse bevacizumab resistance in ETV5 + CRC.

In conclusion, our data revealed ETV5 as a novel biomarker for bevacizumab treatment in CRC. ETV5-mediated CCL2 promotes bevacizumab resistance, and the combination of bevacizumab and anti-CCL2 treatment should be considered as a promising antiangiogenic therapeutic strategy for ETV5 + CRCs.

Abbreviations

CRC: colorectal cancer; TCGA: The Cancer Genome Atlas; GSEA: Gene Set Enrichment Analysis; CAM: chick embryo chorioallantoic membrane; HUVECs: human umbilical vein endothelial cells.

Declarations

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We acknowledge the open databases of TCGA and GEO (Gene Expression Omnibus).

Authors' contributions

Ren Zhao, Kun Liu and Xi Cheng designed and analyzed experimental data. Haoran Feng, Juyong Liang and Changgang Wang performed the experiments. Weihua Qiu and Xi Cheng prepared

Figures. All authors wrote, read and approved the final manuscript.

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Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All aspects of this study were approved by the Research Ethics Committee of Shanghai Jiaotong University.

Consent for publication

Not applicable.

Competing interests

The authors declared no conflict of interest.

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Additional Files

Additional file 1: **Figure S1.** ETV5 directly binds to VEGFA promoter. **a** Diagram of the VEGFA promoter, with the yellow rectangle showing the ETV5 binding sites and red highlighting the mutated ETV5 binding motif in the P2 promoter. **b** ChIP was performed with an anti-ETV5 antibody in RKO cells to analyze ETV5 binding to the VEGFA promoter. RT-PCR experiments were performed using primers against the indicated area (P1 and P2) in the VEGFA promoter, and the indicated region showed significant enrichment compared with the GAPDH control. **c** and **d** Analysis of luciferase activity of the wild type (WT) VEGFA promoter-driven luciferase reporter in RKO cells, or WT and mutant (MUT) VEGFA promoter-driven luciferase reporters in ETV5-overexpressing cells. NC: negative control. PC: positive control. Error bars represent the means of 3 independent experiments, ***p<0.001, ****p<0.0001.

Figures

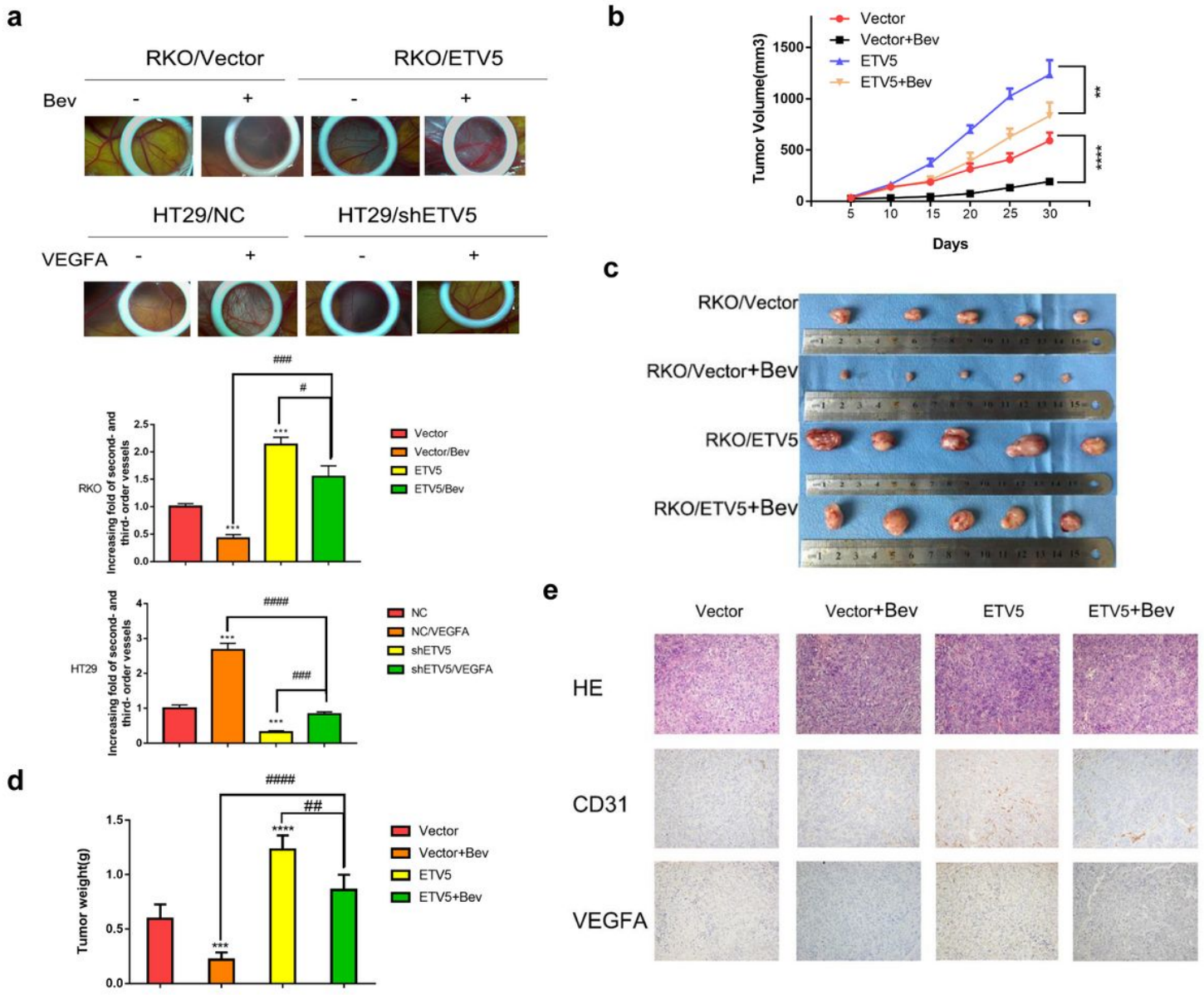

Figure 1

ETV5 upregulation inhibits angiogenesis and bevacizumab resistance in CRC cells through VEGFA in vivo. a The CAM assay was used to examine effect of bevacizumab and VEGFA on blood vessel formation after stimulation with the supernatants from the indicated cells. b Xenografted tumor growth after subcutaneously injecting mice with the indicated cells (n = 5). c Tumor volumes were calculated to measure tumourigenesis ability. d The average tumor weight for each group was calculated. e Representative images of HE staining and IHC staining of CD31 in subcutaneous tumor tissue of RKO/Vector, RKO/Vector+Bev, RKO/ETV5, RKO/ ETV5+Bev group. “*” represents comparing with the control. Bev: bevacizumab. **p<0.01,***p<0.001, ****p<0.0001. # p< 0.05, ## p< 0.01, ### p< 0.001, #### p< 0.0001.

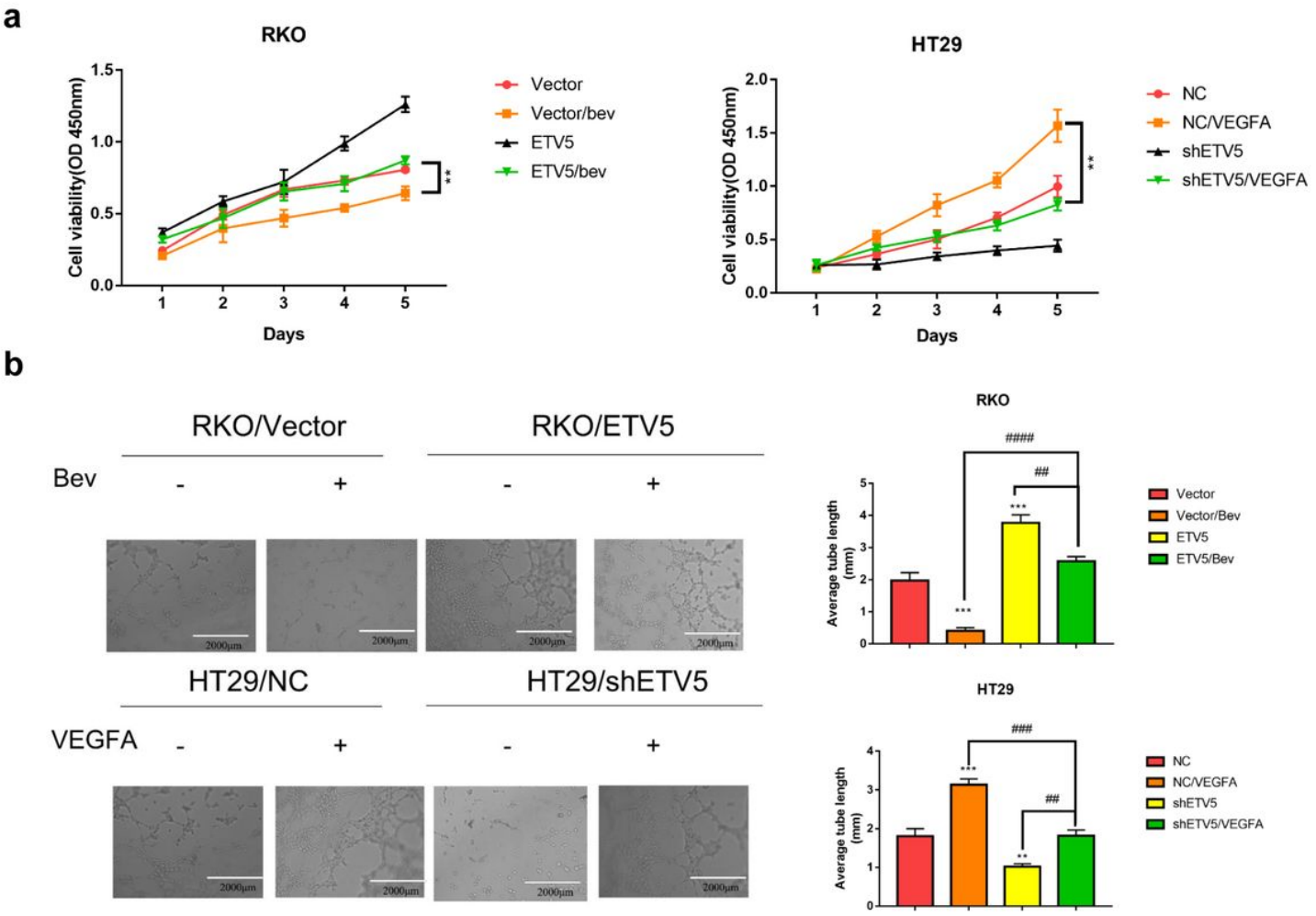

Figure 2

Ectopic overexpression of ETV5 promote bevacizumab resistance in CRC in vitro. a CCK8 was used to measure the effect of bevacizumab or/and recombinant human VEGFA protein on proliferation of HUVECs incubated with conditioned media collected from the indicated CRC cells. b A representative image of the formation of HUVEC tubules following incubation with supernatants collected from the indicated cells and treatment with bevacizumab or/and recombinant human VEGFA protein. Bev: bevacizumab. “*” represents comparing with the control. **p<0.01, ***p<0.001. ## p< 0.01, ###p<0.001, ####p<0.0001.

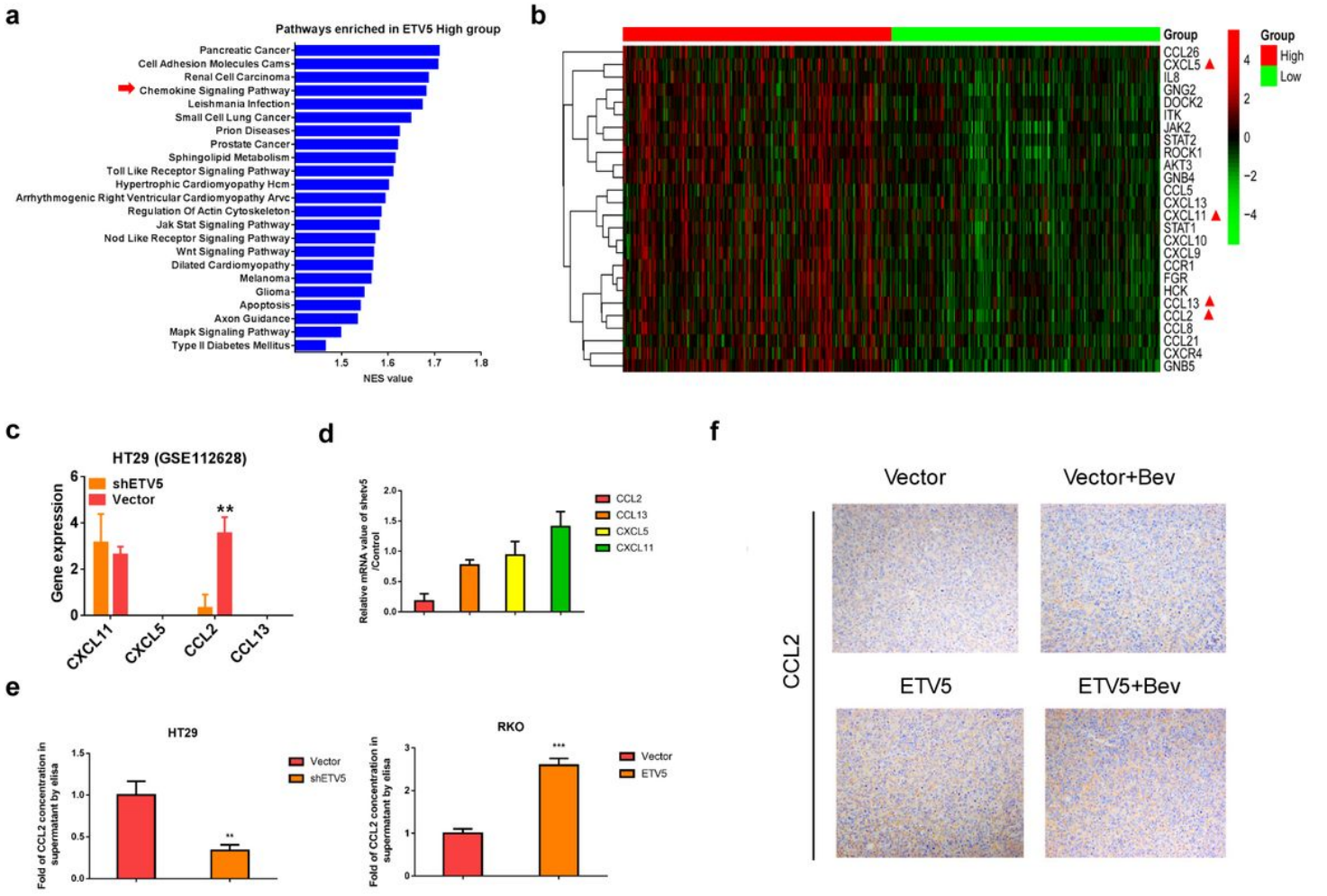

Figure 3

CCL2 is identified as another proangiogenic factor regulated by ETV5 in CRC. a GSEA analysis showed pathways positively related to ETV5 expression in CRC in TCGA database. b Heatmap displayed the significantly enriched genes in chemokine signaling pathway. c Among the significantly enriched genes, four chemokines exhibited proangiogenic role, CXCL5, CXCL11, CCL2 and CCL13. Their expression were compared between HT29/Control and HT29/shETV5 cells (GSE112628). d qRT-PCR was used to examine expression of CXCL5, CXCL11, CCL2 and CCL13 in HT29/shNC and HT29/shETV5 cells. e CCL2 concentrations in the supernatants of HT29/Vector, HT29/shETV5, RKO/Vector, RKO/ETV5 were determined by ELISA. f Representative images of IHC staining of CCL2 in subcutaneous tumor tissue of RKO/Vector, RKO/Vector+Bev, RKO/ETV5, RKO/ ETV5+Bev group. Bev: bevacizumab. “*” represents comparing with the control. **p<0.01, ***p<0.001.

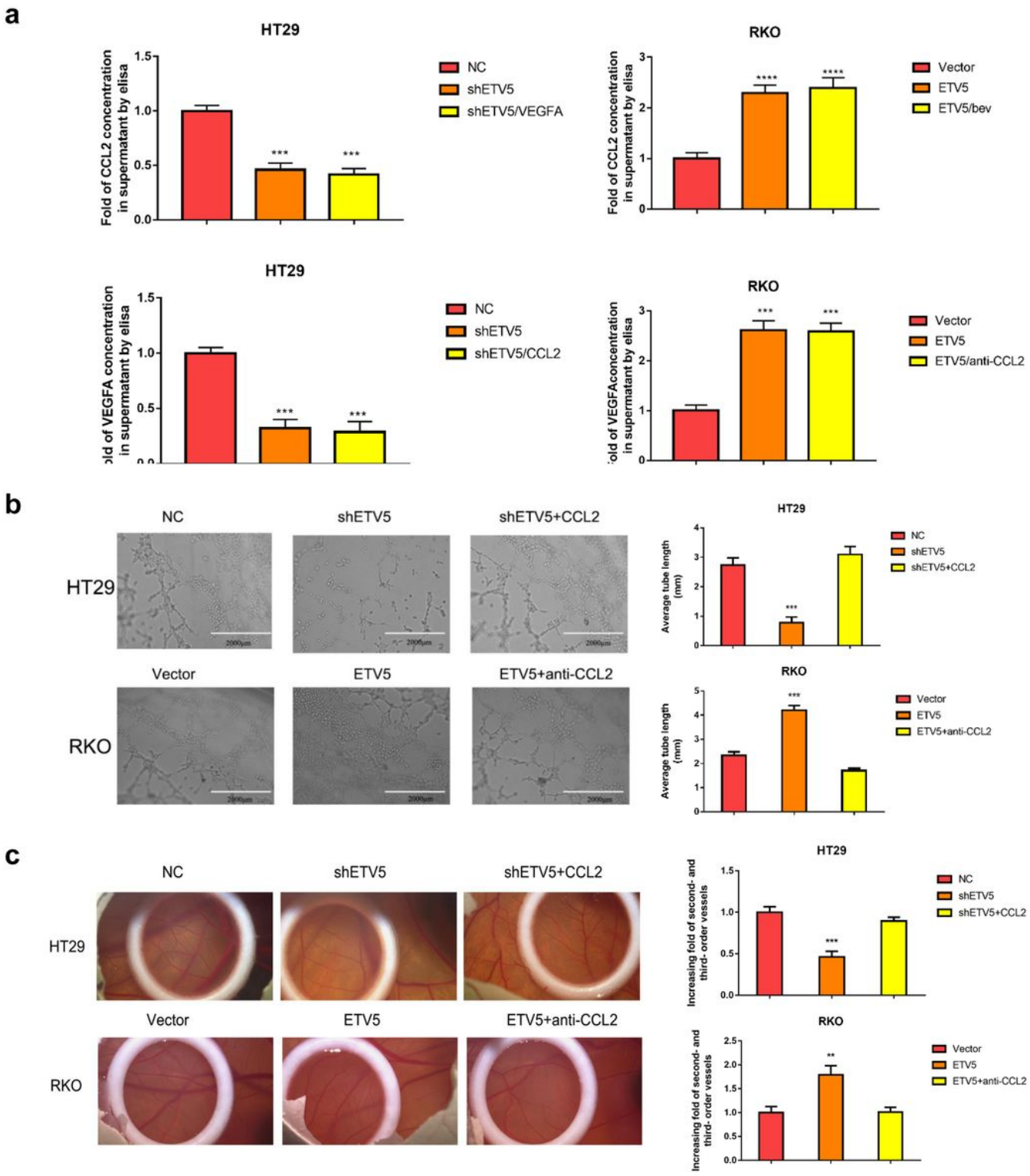

Figure 4

Tumor cell-derived CCL2 partly contributes to ETV5 mediated angiogenesis in CRC. a Recombinant human VEGFA or CCL2 protein did not reversed ETV5 downregulation reduced CCL2 or VEGFA secretion by ELISA respectively. Bevacizumab or anti-CCL2 did not reversed ETV5 upregulation induced CCL2 or VEGFA secretion by ELISA respectively. Bev: bevacizumab. b A representative image of the formation of HUVEC tubules following incubation with supernatants collected from the indicated cells and treatment

with recombinant human CCL2 protein or CCL2 antibody. c The CAM assay was used to examine effect of recombinant human CCL2 protein or CCL2 antibody on blood vessel formation after stimulation with the supernatants from the indicated cells. “*” represents comparing with the control. **p<0.01, ***p<0.001, ****p<0.0001

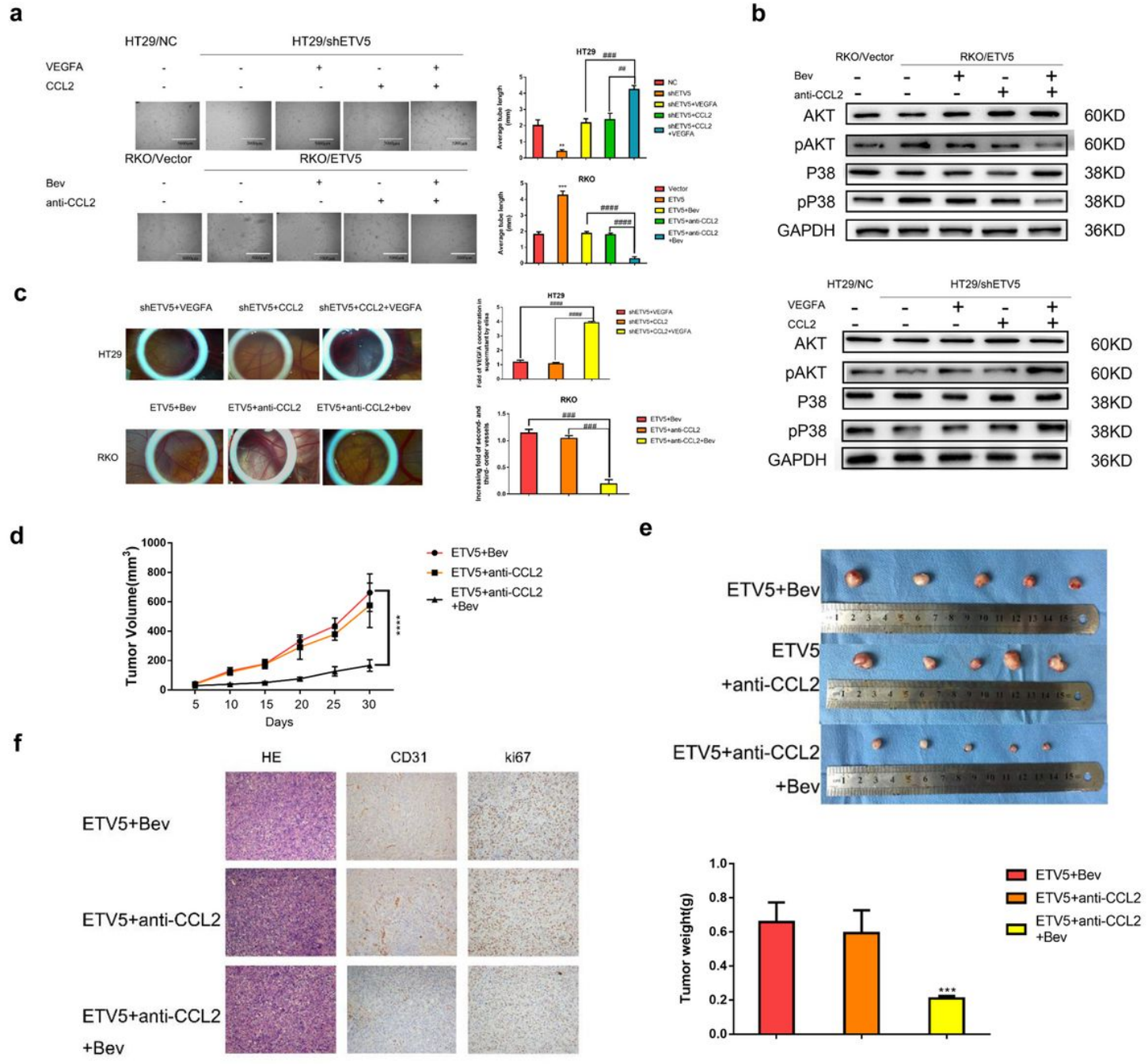

Figure 5

Combination of anti-VEGFA and anti-CCL2 synergistically inhibit tumor growth and angiogenesis in CRC. a A representative image of the formation of HUVEC tubules following incubation with supernatants collected from the indicated cells and treatment with recombinant human VEGFA and CCL2 protein or bevacizumab and CCL2 antibody. b HUVECs were incubated with the indicated supernatants and treated

with recombinant human VEGFA and CCL2 protein or bevacizumab and CCL2 antibody. Activation of the VEGFR downstream signaling pathway was determined by measuring the levels of pAKT and pP38 by Western blot. GAPDH was used as a loading control. c The CAM assay was used to examine effect of recombinant human VEGFA and CCL2 protein or bevacizumab and CCL2 antibody on blood vessel formation after stimulation with the supernatants from the indicated cells. d Xenografted tumor growth after subcutaneously injecting mice with the indicated cells (n = 5). e Tumor volumes were calculated to measure tumourigenesis ability. The average tumor weight for each group was calculated. f Representative images of HE staining and IHC staining of CD31 and ki67 in subcutaneous tumor tissue of RKO/ETV5, RKO/ ETV5+Bev and RKO/ETV5+Bev+anti-CCL2 group. Bev: bevacizumab. “*” represents comparing with the control. **p<0.01, ***p<0.001, ****p<0.0001. ##p<0.01, ###p<0.001, ####p<0.0001.

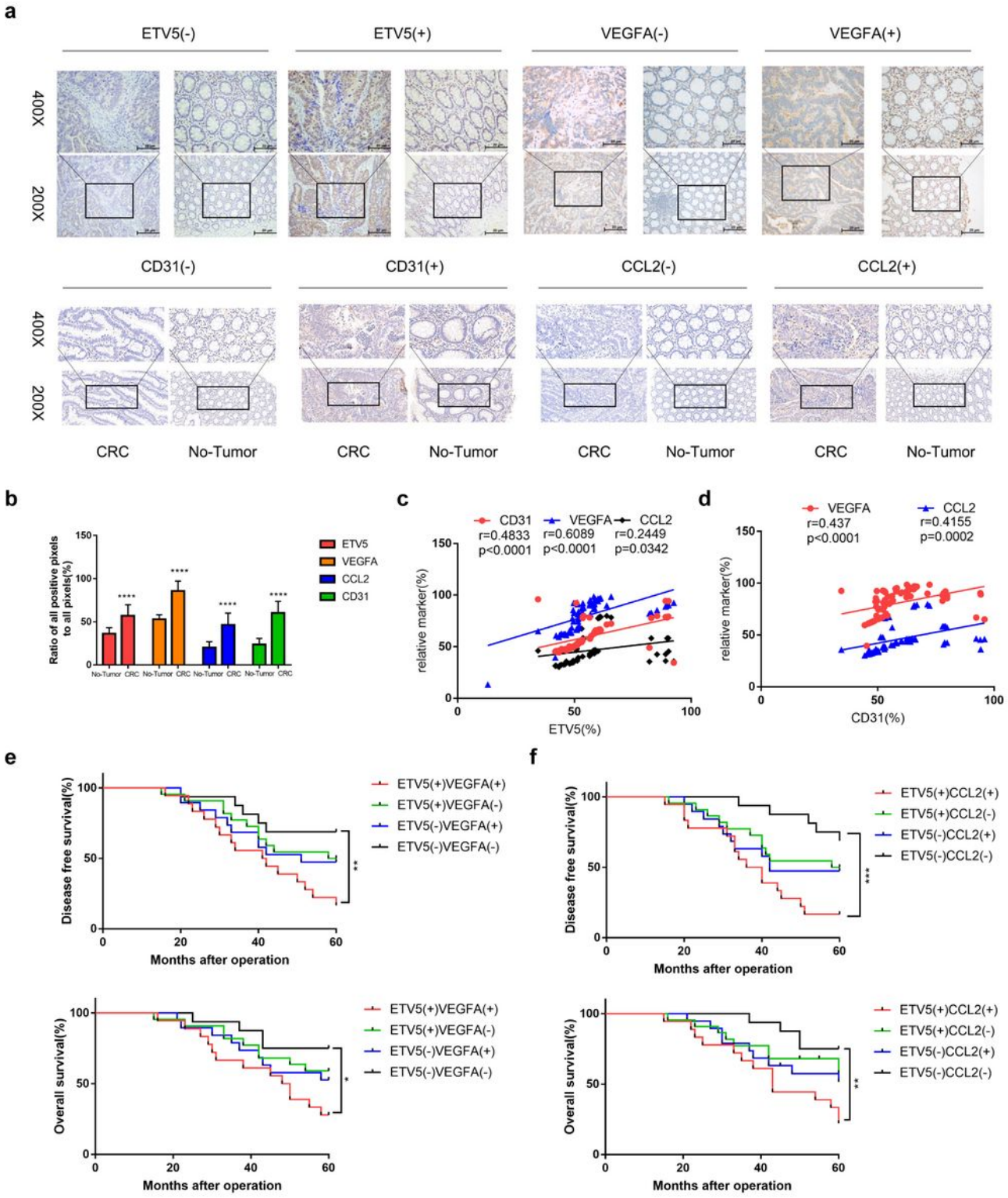

Figure 6

ETV5 expression correlates positively with VEGFA levels in CRC tissues. a Representative images of IHC staining for ETV5, VEGFA, CCL2 and CD31 in the 75-patient cohort. b Expression of ETV5, VEGFA, CCL2 and CD31 was upregulated in CRC tissues than normal colorectal tissues (all p<0.0001). c Expression of ETV5 was positively related to expression of VEGFA (p<0.0001), CCL2 (p=0.0342) and CD31 (p<0.0001) in CRC tissues. d VEGFA (p<0.0001) and CCL2 (p=0.0002) were significantly associated with CD31 in CRC

tissues. e and f Overall survival and disease-free survival curves of the 75 patients in the cohort, as stratified by ETV5 and VEGFA expression pattern or by ETV5 and CCL2 expression pattern. *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

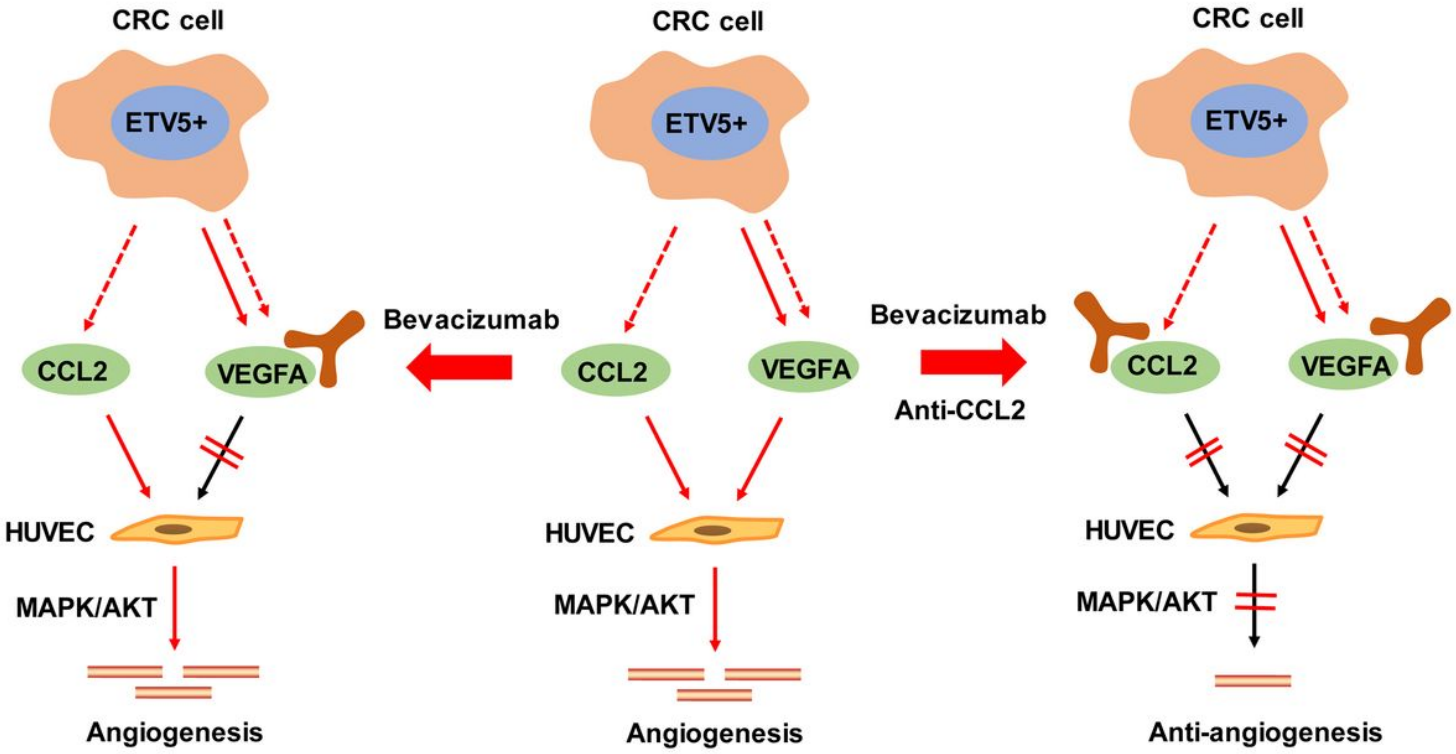

Figure 7

Schematic of ETV5 mediated anti-VEGF therapy resistance in ETV5+ CRC. In CRC, ETV5 could promote angiogenesis via secretion of VEGFA and CCL2. When ETV5+ CRC receiving bevacizumab treatment, paracrine of CCL2 could induce angiogenesis by activating MAPK and AKT pathways in human umbilical vein endothelial cells, which results in bevacizumab resistance. Therefore, combination of bevacizumab and anti-CCL2 treatment could synergistically suppress angiogenesis by simultaneously neutralizing ETV5 induced VEGFA and CCL2 in CRC, and the combined therapy may be promising antiangiogenic strategy for ETV5+ CRCs.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.jpg