



# Multiplex fluorescent immunohistochemistry quantitatively analyses microvascular density (MVD) and the roles of TGF- $\beta$ signalling in orchestrating angiogenesis in colorectal cancer

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**Background:** Advances in multiplex fluorescent immunohistochemistry (mfIHC) techniques and digital pathology platforms allow the quantification of multiple proteins in the same tissue section and produce continuous data. Previously, we used mfIHC to establish the expressed profiles of proteins involved in TGF- $\beta$  signalling in colorectal cancer (CRC).

**Methods:** We used mfIHC to show microvascular density (MVD) by staining CD31 in the tissues from CRC patients. We further investigated the relationship between MVD and TGF- $\beta$  signalling.

**Results:** We found that the levels of MVD were significantly higher in cancer tissues than in paired normal tissues. Prognostic analysis revealed that the survival time for CRC patients with high levels of MVD was significantly shorter than that for those with low levels of MVD. Systematic analysis of the levels of MVD and TGF- $\beta$  signalling proteins revealed that TGF- $\beta$  signalling showed contradictory roles in sustained tumour angiogenesis. In CRC cells, the expression of VEGFA was increased by low concentrations of TGFB1 but decreased by high concentrations of TGFB1. Vessel-forming assays demonstrated that low-dose TGFB1 stimulated but high-dose TGFB1 inhibited HUVECs to form vessel tubes.

**Conclusions:** Our analysis based on mfIHC staining in CRC tissues supports the concept that TGF- $\beta$  signalling either promotes or inhibits tumour angiogenesis.

**Keywords:** Colorectal cancer (CRC); angiogenesis; microvascular density (MVD); TGF- $\beta$  signalling

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## Introduction

Colorectal cancer (CRC) is one of the most common malignant tumours (1). Angiogenesis provides oxygen and nutrients to and removes metabolic waste from tumour cells (2). Increased angiogenesis has been associated with neoplastic progression, metastasis and outcome in a number of malignancies (3). Angiogenesis is regulated by multiple growth factors, including VEGF, PDGF, and FGF (4,5). Microvascular density (MVD) is considered the 'gold standard' for measuring tumour angiogenesis (6). Many

studies have shown that MVD could have potential value as a prognostic biomarker of tumours (7). CD31 is recognized as a biomarker of vascular endothelial cells (ECs) to represent MVD in tumour tissues (8).

The transforming growth factor (TGF)- $\beta$  signalling pathway is an intriguing cytokine signalling pathway that exhibits dual activities in malignant tumour disease (9). TGF- $\beta$  exhibits multifunction and acts as an important mediator of cancer angiogenesis (10,11). Research regarding the consequences of TGF- $\beta$  signalling remains

controversial. A large amount of data supports an early tumour-suppressive role for TGF- $\beta$ . Additionally, TGF- $\beta$  signalling within carcinoma cells can promote tumour progression and metastasis at late stages (12). Previously, we conducted a study to detect the multiple factors involved in TGF- $\beta$  signalling in the tissues of patients with CRC by multiplex fluorescent immunohistochemistry (mFIHC) (9). Our results supported the idea that TGF- $\beta$  exhibited tumour-suppressive effects at the early stage and promoted tumour progression at the late stage.

TGF- $\beta$  consists of three isoforms (TGFB1,  $\sim$ 2, and  $\sim$ 3). TGFB1 is the most abundant and well-studied isoform (13). There are two typical TGF- $\beta$  receptors, TGF- $\beta$  type I and type II receptors (TGFBR1 and TGFBR2), with active serine/threonine kinase (14). Activated TGF- $\beta$  can bind to TGFBR1/2, which initiates canonical SMAD pathways by phosphorylating SMAD2/3 (15,16). Then, SMAD4 is recruited by phosphorylated-SMAD2/3 and translocates to the nucleus to activate TGF- $\beta$ -responsive target genes (17). SMAD1/5/9 are targets of the bone morphogenetic protein (BMP) receptor, regarded as non-canonical SMAD-independent pathways (18). SMAD4 coupling with phosphorylated SMAD1/5/9 can mediate antitumour functions (19). Typically, TGF- $\beta$  inhibits cell proliferation by arresting cells in the G1 phase through increasing the expression of p15 and p21 (20). In cancer cells, the overexpression or genomic mutations of oncogenes reversed the functions of TGF- $\beta$  by downregulating p15 and p21 (21). TGF- $\beta$  signalling participated in tumour angiogenesis by inducing a proangiogenic environment (22).

TGF- $\beta$  is one of the major regulators of extracellular matrix proteins in vascular cells (23). TGF- $\beta$ 1 has been shown to enhance angiogenesis by upregulating VEGF and recruiting more ECs (24). However, the inhibited effects of TGF- $\beta$  signalling on cancer cells did not match the role of pro-angiogenesis in the early stage of tumorigenesis. We thus deduced that TGF- $\beta$  signalling would exhibit dual roles in orchestrating tumour angiogenesis. Here, we attempted to analyse the relationships between MVD and TGF- $\beta$  signalling. By mFIHC staining, we established a dataset of eight quantitatively elevated TGF- $\beta$  signalling proteins (TGFB1, TGFBR1, TGFBR2, SMAD4, SMAD2/3, p-SMAD2/3, SMAD1/5/9, and p-SMAD1/5/9) in the tissues of CRC patients (9). To address the relevance of tumour vessels and TGF- $\beta$  signalling proteins, we further stained CD31 to represent the MVD of each sample using the same method and inferred the clines of the dual roles of TGF- $\beta$  signalling. In addition, we confirmed that

the activation of TGF- $\beta$  signalling could promote tumour angiogenesis in cancer cells, while the excessive activation of TGF- $\beta$  signalling could prevent tumour angiogenesis.

## Methods

### Patients

The HCol-Ade180Sur-07 tumour tissue microarray (Shanghai OUTDO Biotech Co., Shanghai, China) consisted of 90 paired colorectal adenocarcinoma tissues and matched normal mucosa (25). Patients underwent surgery from January 2009 to October 2009, and follow-up information was available from February 2009 to May 2014. All sample donors provided informed consent, and the study was conducted under the approval of the Institutional Ethics Committee of Beijing Chaoyang Hospital of Capital Medical University. All tissue samples were collected from patients with colon cancer for whom chemotherapy or radiotherapy was not received before surgery. All procedures were performed in accordance with the relevant guidelines and regulations. All CRC patients were recruited based on the histopathology results. Tumour stage was determined according to the National Comprehensive Cancer Network (NCCN) criteria.

### Immunostaining of TMA

The TMA slides were stained using the PerkinElmer OpalTM 4-colour FIHC Kit (Cat#, NEL80001KT, PerkinElmer, Hopkinton, MA, USA). Briefly, the deparaffinization protocol was run (xylene 15 min two times, 100%, 95%, 85%, and 75% ethanol for 10 min) until rinsing in water for 10 min. Then, the slides were pretreated with antigen retrieval solution (provided in the Opal kit) by microwaving method (45 seconds on 100% power, followed by 15 min on 20% power), followed by 10 min blocking in 10% goat serum in TBST and rinsing in water. Then, the TMA slides were stained with a primary antibody against CD31 (dilution, 1:1,000, Cat#: ZA0568, Zhongshan Golden Bridge, Beijing, China) for 1 hour at room temperature. After washing the slides three times with 1X TBST, the slides were incubated with horseradish peroxidase-labelled rabbit secondary antibody (Cat#: SAP9001, Zhongshan Golden Bridge, Beijing, China) for 10 min. A tyramide-conjugated fluorophore (Opal520) was added to the slides at a 1:100 dilution in Amplification Plus Buffer and incubated for 10 min at RT. The conjugated antibody was removed by microwave. The TMA slides were further stained with a

primary antibody against SMAD4 (dilution 1:2,000, Cat#: sc-7966, Santa Cruz) for 1 hour at room temperature. After washing with 1X TBST, the slide was subjected to labelling with a tyramide-conjugated fluorophore (Opal620), and the microwave removed the conjugated antibody. Finally, the slide was counterstained with Spectral DAPI to show nuclei and cover slips, followed by sealing with mounting medium supplied with antifade solution (Applygen Technologies Inc. Beijing, China).

#### ***Quantitatively calculated MVD by multispectral images***

InForm 2.1.1 software (Perkin Elmer) was used for the batch analysis of multispectral images from the experiment. The images were loaded into InForm to build a single colour compensation library and substrate background using the image derived from a slide without staining in the same exposure settings. To calculate the number of MVD, three to six representative regions of interest for high-powered (200 $\times$ ) imaging from every case were selected. To build algorithms for segment tissues and cells, a few representative multispectral images from the experiment were loaded into InForm software. Then, the segmented tissues of parenchymal neoplasms and mesenchyme and cells were trained according to the 4',6-diamidino-2-phenylindole (DAPI) signal intensity. Next, the detected tissue compartments were selected and quantified for each stained protein on the slides. InForm software was used to quantitatively measure the number of CD31-stained MVD. All remaining multispectral images from the experiment were batch processed.

#### ***Western blotting***

In all, 30  $\mu$ g of cell lysates was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The membranes were blocked with 5% milk and then incubated with various primary antibodies at 4 °C overnight. The antibodies were directed against the following: VEGFA (dilution 1:500, Cat#: ab46154, Abcam, Cambridge, MA, USA), TGFB1 (1:100, Cat#: 3711, CST, Danvers, MA, USA), SMAD4 (1:500, Cat#: sc-7966, Santa Cruz, CA, USA), TGFBR1 (1:200, Cat#: ab135814, Abcam), and TGFBR2 (1:400, Cat#: ab78419, Abcam). Then, the membranes were incubated with the IRDye 680RD goat anti-mouse/rabbit IgG secondary antibodies at 1:10,000 dilutions (Cat#:926-68070/68071, LI-COR, NE, USA).

#### ***HUVEC Matrigel tube-forming assay***

Initially, Matrigel (Cat#:356234, BD Biosciences) was polymerized (200  $\mu$ L/well of a 48-well culture plate) for 30 min at 37 °C. Serum-starved HUVECs were resuspended in endothelial cell medium (ECM) media and reseeded onto Matrigel-coated wells (105 cells/well). The human TGFB1 protein [Cat#: 5154, cell signaling technology (CST)] was incorporated into the ECM and cultured with HUVECs. After 24 hours, images of capillary-like networks were recorded using a phase-contrast microscope. The images were analysed with the Angiogenesis Analyzer plugin for ImageJ (Carpentier *et al.*, Angiogenesis Analyzer for ImageJ. 4th ImageJ User and Developer Conference Proceedings).

#### ***TCGA dataset analysis and statistics***

Molecular data from human colon cancers (COAD) were generated by the TCGA Research Network (<http://www.cbioportal.org/>). The significance of the data from patient specimens was determined by the Mann-Whitney U test. Overall survival (OS) rates were assessed by the Kaplan-Meier test, and the log-rank test was used to plot survival curves.  $P < 0.05$  was considered significant.

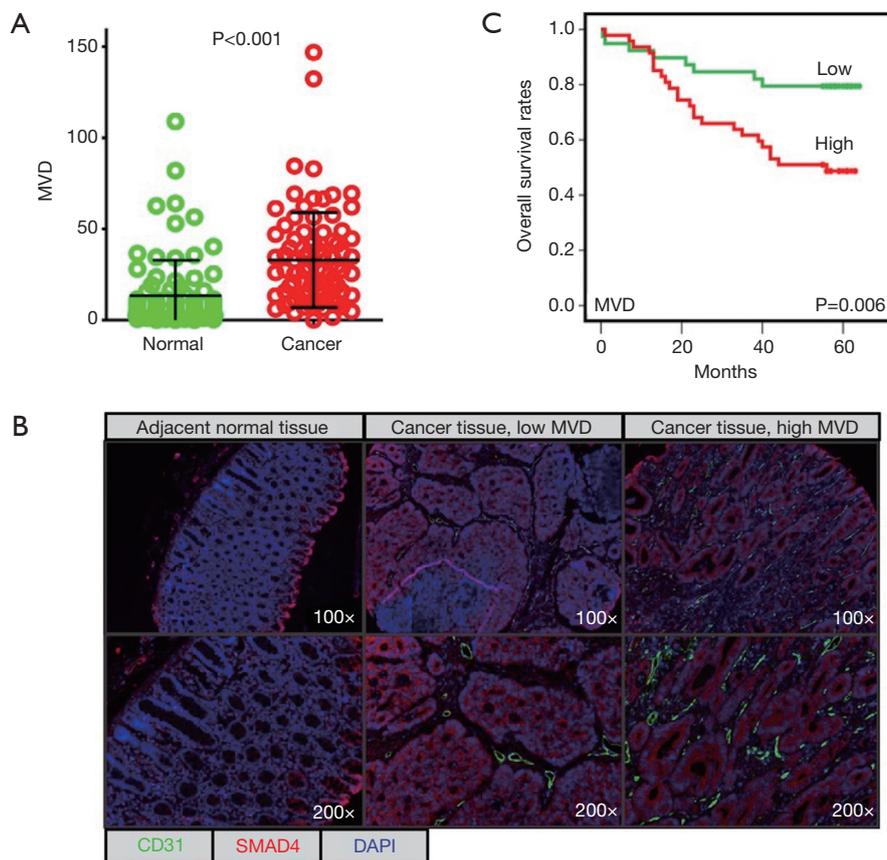
## **Results**

#### ***mfiHC stains CD31 in the tissues of CRC***

mfiHC was used to stain CD31 in 90 paired CRC tissues (cancer *vs.* adjacent normal tissues). We explored a scoring system based on InForm software to quantitatively evaluate CD31-stained MVD in CRC cancer tissues. As expected, the MVD was significantly higher in cancer tissues (means  $\pm$  SD, 32.96 $\pm$ 26.01) than in normal tissues (13.40 $\pm$ 19.45) (*Figure 1A*). Tumour microvasculature is one of the most important components of the tumour microenvironment. mfiHC showed the microvasculature located in the stroma of cancerous tissues (*Figure 1B*). In comparison, SMAD4-stained cancer cells were previously demonstrated to be expressed by cancer cells (9). Our results also demonstrated that high MVD (n=47) for CRC patients predicted a shorter OS time than those patients with low MVD (n=39) (*Figure 1C*).

#### ***MVD associates with advanced clinical pathological features***

Next, we analysed the relationships between MVD and clinical pathological characteristics. The CRC patients were



**Figure 1** mfiHC stains CD31 to show the microvessels in the tissues of colon cancer. (A) The MVD between cancer tissues and adjacent normal tissues were compared; (B) representative images show CD31-stained MVD for adjacent normal tissues, low MVD, and high MVD in cancer tissues. SMAD4 was stained to label the tumour cells; (C) Kaplan-Meier analysis of OS rates. mfiHC, multiplex fluorescent immunohistochemistry; MVD, microvascular density; OS, overall survival.

characterized by tumour size ( $\leq 3$  vs.  $> 3$  cm), TNM stage (I–II vs. III–IV), differentiation (high vs. moderate vs. low), and lymph node status (negative vs. positive) (Figure 2A). Although there were no statistically significant differences, CRC patients with a higher degree of malignancy (tumour size  $> 3$  cm, TNM III–IV, poor differentiation, positive lymph node) were found to have higher MVD.

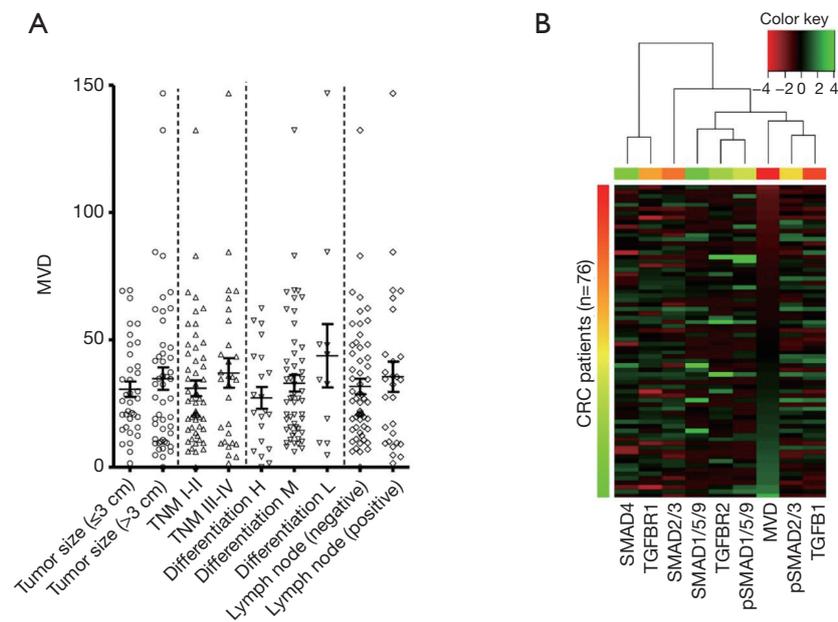
#### *mfiHC analysis reveals the dual effects of TGF- $\beta$ signalling on regulating angiogenesis*

Unsupervised hierarchical clustering was used to explore the relationships between MVD and TGF- $\beta$  signalling (Figure 2B). The MVD was ordered from low to high and clustered with pSMAD2/3 and TGFB1 into a subgroup. However, this analysis did not successfully divide TGF- $\beta$

signalling into significantly expressed clusters. Generally, TGFB1 and pSMAD2/3 were positively associated with MVD in patients with low MVD. However, TGFB1 and pSMAD2/3 were negatively associated with MVD in patients with high MVD. Based on these findings, we speculated that TGF- $\beta$  signalling also exhibited dual effects on orchestrating tumour angiogenesis in CRC.

#### *A low dose of TGFB1 increases VEGFA expression but a high dose of TGFB1 inhibits VEGFA expression*

VEGFA is recognized as the most important angiogenic cytokine and serves as a target of anti-tumour agents, such as sorafenib. To determine the effects of TGF- $\beta$  signalling on the expression of VEGFA, we used human recombinant TGFB1 proteins to treat HCT116 and SW480 cells. In



**Figure 2** The relationships of MVD, pathological features, and TGF- $\beta$  signalling. (A) Dot plots compare the levels of MVD for colon patients with tumour size, TNM stage, differentiation (H: high vs. M: moderate vs. L: low), and lymph node; (B) heatmap clusters the MVD and TGF- $\beta$  signalling proteins. MVD, microvascular density.

both HCT116 and SW480 cells, TGFB1 increased the expression of VEGFA when used at a relatively low dose (30~90 ng/mL) (Figure 3A). However, when 120 ng/mL TGFB1 was used to treat HCT116 and SW480 cells, the VEGFA levels decreased (Figure 3B).

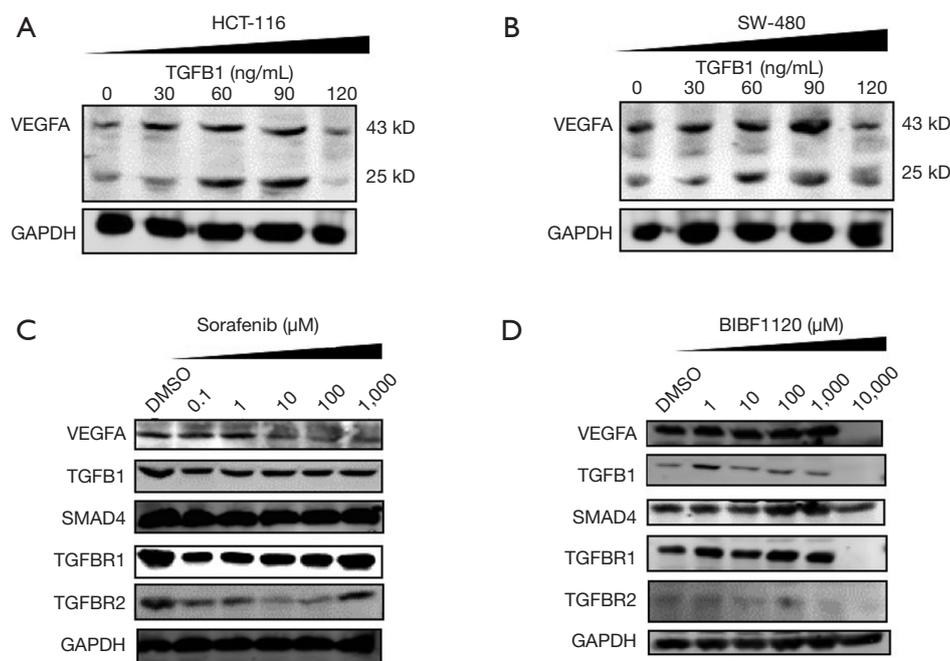
#### **Angiogenic inhibitors either promote or inhibit TGF- $\beta$ signalling**

The strategy of targeting the VEGFA pathway to inhibit tumour progression has been widely used in clinical practice for the treatment of advanced CRC patients. However, the effectiveness of these drugs was limited. We speculated that the dual roles of TGF- $\beta$  signalling might contribute to changes in the anti-angiogenic effects of those drugs. To verify this hypothesis, we detected VEGFA and proteins involved in TGF- $\beta$  signalling after stimulating HCT116 cells with a series of concentrations of sorafenib for 24 hours. The levels of VEGFA were decreased in a dose-dependent manner (Figure 3C). TGFB1, TGFB1, and TGFB2 were decreased when treated with 0.1  $\mu$ M sorafenib. Subsequently, TGFB1 and TGFB1 were increased after treatment with 1, 10, 100, and 1,000  $\mu$ M sorafenib. TGFB2 was increased under stimulation with

1,000  $\mu$ M sorafenib. In addition, BIBF1120 is an inhibitor of VEGFR and was administered to HCT116 cells at various concentrations (Figure 3D). BIBF1120 did not change the levels of VEGFA when the concentrations were less than 1,000  $\mu$ M. However, a high dose of BIBF1120 (10,000  $\mu$ M) deleted the expression of VEGFA, TGFB1, TGFB1, and TGFB2. Moreover, low-dose BIBF1120 (<1,000  $\mu$ M) increased the levels of TGFB1 and TGFB1. Both sorafenib and BIBF1120 failed to change the levels of SMAD4.

#### **Excessive TGFB1 inhibited HUVECs to form tubes-like structures**

Next, HUVEC Matrigel tube-forming assays were applied to determine the effects of TGFB1 on angiogenesis. HUVECs were seeded onto Matrigel-coated wells with media containing human recombinant TGFB1 proteins. The media with 30 ng/mL TGFB1 increased HUVECs to form tube-like structures when compared with the treatment in which HUVECs were cultured with media without TGFB1. However, media with 300 ng/mL TGFB1 did not enhance the ability of HUVECs to form tubes (Figure 4A). The number of branches and total length of



**Figure 3** TGF- $\beta$  signalling regulates the expression of VEGFA. TGF $\beta$ 1 increased the levels of VEGFA (43 and 25 kD) when cultured HCT-116 (A) and SW-480 (B) cells in media containing TGF $\beta$ 1 at relatively low concentrations (30–90 ng/mL). However, high concentrations of TGF $\beta$ 1 (120 ng/mL) decreased the levels of VEGFA. The angiogenetic inhibitors sorafenib (C) and BIBF1120 (D) decreased the levels of TGF $\beta$ 1, TGFB1, and TGFB2 by low dose but increased their levels by high dose in HCT116 cells.

the HUVEC-forming tube were calculated after treatment with media supplied with none, low-, or high-dose TGF $\beta$ 1 (Figure 4 B,C). The branches and lengths were significantly increased by treatment with low-dose TGF $\beta$ 1 but were decreased by treatment with high-dose TGF $\beta$ 1.

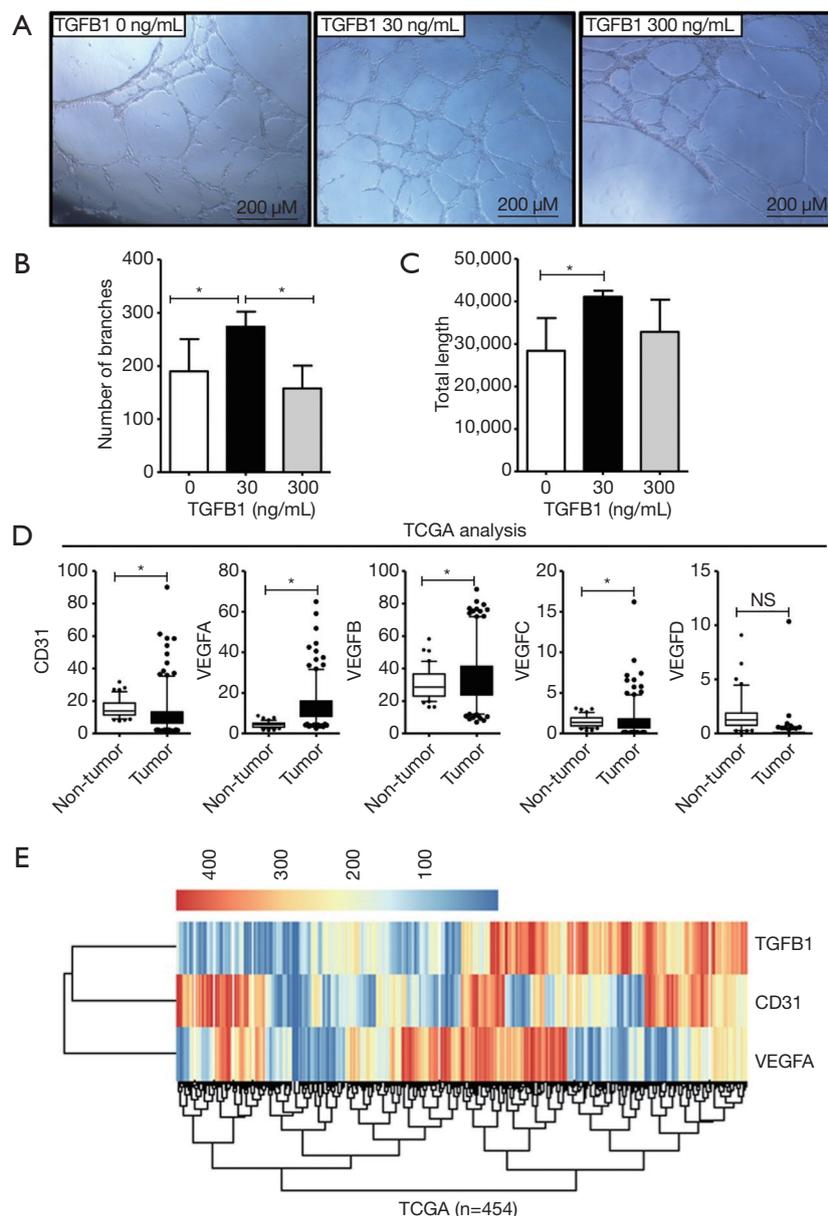
Next, we analysed the differential expression of genes in the TCGA COAD datasets, which contained the normalized values of colon cancer tissues by RNA-sequencing. Here, we compared the mRNA levels of CD31 and the VEGF family (including VEGFA, VEGFB, VEGFC, and VEGFD) between tumour tissues (n=454) and non-tumour tissues (n=41) (Figure 4D). CD31, VEGFA, VEGFB, and VEGFC showed significantly higher expression in tumour tissues than in non-tumour tissues ( $P < 0.05$ ). Unsupervised hierarchical clustering was further used to analyse the expression profiles of the mRNA levels of CD31, VEGFA, and TGF $\beta$ 1 (Figure 4E). These data did not reveal significant correlations in expression. In detail, CRC patients with high TGF $\beta$ 1 levels included patients with either high or low CD31 or VEGFA levels, and vice versa. The profiles of CD31 and TGF $\beta$ 1 were similar, and mflHC identified patterns for CD31 and TGF $\beta$ 1 on

protein levels. The profiles of VEGFA and CD31 also failed to cluster the significantly positive or negative correlation patterns on mRNA levels. The above data partially supported our conclusion that low activated TGF- $\beta$  signalling enhanced tumour angiogenesis, but excessive TGF- $\beta$  signalling abolished tumour angiogenesis.

## Discussion

Both our previous study (9) and another study (26) revealed hyperactive TGF- $\beta$  signalling in CRC. The mflHC allowed us to quantitatively analyse the relationships between TGF- $\beta$  signalling and tumour angiogenesis in CRC tissues. The present study provides an opportunity to consider the novel application for proteins involving TGF- $\beta$  signalling that are used as biomarkers to conduct antiangiogenic therapy.

As noted, TGF- $\beta$  signalling plays contradictory roles in cancer and has been extensively explored by many studies (16,27). This paradox is reflected in the clinic, where in early stage cancers, the levels of TGF- $\beta$  are positively associated with a favourable prognosis.



**Figure 4** TGFB1 contributes to the HUVEC tube forming assay and TCGA analysis. (A) Representative pictures of the HUVEC tube forming assays. HUVECs were treated with TGFB1 (0, 30, and 300 ng/mL), and each treatment was replicated three times; (B,C) the number of branches and total length of HUVEC-forming tubes were calculated and compared after treatment with a dose series of TGFB1; (D) TCGA database (COAD) analysed the mRNA levels of CD31, VEGFA, VEGFB, VEGFC, and VEGFD in patients with colon cancer. The data included 454 cancer tissues and 41 non-tumour tissues; (E) heatmap shows the expression of CD31, VEGFA, and TGFB1 in 454 cancer tissues. NS, not significant. \*, P<0.05.

However, in advanced tumours, the levels of TGF- $\beta$  are positively associated with tumour size, invasiveness, and dedifferentiation (28). However, few studies have reported the contradictory roles of TGF- $\beta$  signalling in orchestrating

tumour angiogenesis. Normally, angiogenesis is tightly controlled by a balance of angiogenic and antiangiogenic factors. VEGF is recognized as the most critical and specific factor that stimulates both physiological and pathological

angiogenesis (29). TGF- $\beta$  expression was positively and substantially associated with VEGF expression and angiogenesis in late-stage CRC (30). Tumour-derived TGF- $\beta$  was proven to regulate the proliferation and migration of vascular ECs (31). TGF- $\beta$  was proven to bind ALK5 and ACVRL1, both of which exhibited antagonistic effects (32). Furthermore, at high concentrations of TGF- $\beta$ , the canonical SMAD2/3 pathway is activated by ALK5, inducing the expression of plasminogen activator inhibitor and fibronectin, thereby impeding angiogenesis (33). At low concentrations, TGF- $\beta$  promoted EC cell proliferation and migration by enhancing the expression of MMPs through ALK1 activation of SMAD1/5 (34). In the present study, mfiHC staining supported that TGF- $\beta$  signalling showed positive roles at low activity in stimulating angiogenesis and increasing VEGFA expression. However, excessive TGF- $\beta$  signalling acted as a negative regulator of angiogenesis and decreased VEGFA expression. This phenotype turnover might be the reason that the hyperactivation of TGF- $\beta$  signalling prolonged the OS time by inhibiting tumour angiogenesis in patients with CRC.

VEGF is a factor of angiogenesis that includes several alternative isoforms with significantly different functions (2). VEGF165 antagonized VEGF165b-induced EC proliferation and competitively bound to VEGFR2 (35). Bevacizumab is a humanized monoclonal antibody against VEGF and has been used for CRC therapy by blocking all angiogenic isoforms of VEGF (36). The polymorphisms of VEGF have been found to be correlated with the efficacy of bevacizumab (37). Moreover, bevacizumab was proven to be less effective in obese metastatic CRC patients (38). The genetic variations of TGFBI did not find any relationships with the outcomes for CRC patients who received bevacizumab (39). In contrast, our present study provides evidence that the protein levels for those factors involved in TGF- $\beta$  signalling might contribute to predicting the efficacy of bevacizumab-based chemotherapy.

In conclusion, our study is the first to report the dual roles of TGF- $\beta$  signalling in orchestrating tumour angiogenesis in CRC. mfiHC provides the ability to simultaneously and quantitatively analyse TGF- $\beta$  signalling and MVD. The main finding shows that low active TGF- $\beta$  signalling enhances angiogenesis, but hyperactive TGF- $\beta$  signalling inhibits angiogenesis. Further mechanistic studies will need to address the hyperactive TGF- $\beta$  signalling that abolishes angiogenesis and benefits chemotherapy by anti-angiogenic drugs.

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## Footnote

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.02.09>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All sample donors provided informed consent, and the study was conducted under the approval of the Institutional Ethics Committee of Beijing Chaoyang Hospital of Capital Medical University (No. 2018-ke-24). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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