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ORIGINAL ARTICLE

Analysis of expression levels of markers associated with tumor proliferation and angiogenesis in familial adenomatous polyposis

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Abstract

Background: Familial adenomatous polyposis (FAP) is an autosomal dominant hereditary disease with colorectal adenomatous polyps as the main clinical manifestations. The objective of this study was to analyze and compare the expression levels of tumor proliferation and angiogenesis-related genes in different tissue sections of FAP patients through qPCR, western blot, and immunohistochemistry (IHC) analysis.

Methods: Seventeen patients with FAP admitted to Tianjin Union Medical Center from January 2010 to June 2015 were selected, and then, normal intestinal mucosa, polyp tissue, or cancerous polyp tissue were collected. QPCR, western blot, and IHC were used to detect the expression level of genes or proteins correlated with tumor proliferation.

Results: The mRNA expression of *CD31* in large polyp tissue was significantly higher than that in normal tissue and small polyp tissue. Compared with normal tissue and polyp tissue, the expression level of *KI67* mRNA in cancer tissue was remarkably increased. The *VEGFA* mRNA and *CDH5* mRNA expression in both polyp and cancer tissues were prominently lower than those in normal tissue. The expression of CD31 protein in cancer tissue was lower than that in normal tissue and polyp tissue, whereas the expression levels of VEGF, CDH5, and KI67 protein were widely higher than that in normal tissue and polyp tissue.

Conclusion: Abnormal expressions of CD31, KI67, VEGF(A), and CDH5 were associated with the carcinogenesis of FAP.

KEYWORDS
CD31, CDH5, familial adenomatous polyposis, KI67, VEGF(A)

Zhao Zhang and Dan Wang contributed equally as co-first authors.

1 | INTRODUCTION

Familial adenomatous polyposis (FAP) is an autosomal dominant genetic disorder characterized by a mass of adenomatous polyps in the colon and rectum. FAP is an extremely malignant hereditary disease, the prevalence of newborns is 1:8000 to 12,000, and prevalence of the general population is 1:24,000. FAP patients manifest as not only colorectal polyps, but also a series of additional intestinal manifestations (Smith et al., 2013). The main pathological characteristic of FAP is the widespread distribution of small intestinal mucosal adenomas. These adenomas are densely packed or arranged in groups, hundreds or even thousands in general. FAP patients were born without knots and rectal polyps, most of them develop polyps at the age of 15, and the number increases with age. Without treatment, all patients with this syndrome will develop colon cancer over the ages of 35 and earlier than normal colon cancer (Waller et al., 2016). Additionally, there is an increased risk of progression to other malignancies.

About 70%–90% of FAPs are cancer syndrome caused by germline mutations of adenomatous polyposis coli (APC) gene (DE Marchis et al., 2017). Nevertheless, in addition to the apparent loss of APC function, little is known about the molecular processes of adenoma initiation (Bowden et al., 2007). The cell proliferation is a significant feature in the development of FAP, and the unrestricted proliferation can result in malignant canceration Biasco, 2004). *KI67* (GenBank: AJ567757.1, OMIM: 176741) is a proliferation marker, which represents cell proliferation activity. It expresses in all stages of cell proliferation (G1, S, G2, and M), but not in cell stationary phase (G0). Moreover, it is strongly related to the degree of differentiation, invasion, metastasis, and prognosis of many tumors (Ciesielska et al., 2017; Matsuse et al., 2017; Pan et al., 2017).

Furthermore, clinical treatment of FAP is usually carried out by inhibiting the proliferation of tumor cells (Aihara et al., 2014). In numerous malignant tumors, the angiogenesis is dense and growing rapidly, which plays a crucial role in tumor development and metastasis (Bjerkvig et al., 2009). Angiogenesis mediated by angiogenic factors not only provides nutrition for tumor growth, but also increases the chance of tumor cells entering the circulation and metastasis (Zuazo-Gaztelu & Casanovas, 2018). Among them, vascular endothelial growth factor A (*VEGFA*, GenBank: KJ892374.1, OMIM: 192240) plays an important role in the regulation of angiogenesis signaling pathway. Cadherin 5 (*CDH5*/*VE-cadherin*, GenBank: KJ901329.1, OMIM: 601120) is essential for maintaining and controlling endothelial cell contact, controlling vascular permeability and leukocyte extravasation (Vestweber, 2008). In addition, *CDH5* regulates cell proliferation and apoptosis, and regulates VEGF function. Platelet endothelial cell adhesion molecule-1 (*PECAM1* / *CD31*, GenBank: M28526.1, OMIM: 173445) is a membrane glycoprotein used in immunohistochemistry (IHC) to evaluate tumor angiogenesis. Its activity is mediated by regulating tumor microenvironment (TME) and promoting tumor cell proliferation (Valsamma et al., 2018). Moreover, inhibiting angiogenesis will apparently prevent the development and reproduction of tumors (Lin et al., 2016). Consequently, we speculated that genes related to cell proliferation and angiogenesis might be involved in the development of FAP.

Hence, in this study, we analyzed the gene and protein expression levels of KI67, VEGFA, CD31, and CDH5, which connected with tumor proliferation and angiogenesis in FAP, to explore the potential mechanism of these genes in the carcinogenesis of FAP.

2 | MATERIALS AND METHODS

2.1 | Ethical statement and sample collection

This study was approved by the ethics committee of Tianjin Union Medical Center, China. All research processes were in accordance with the requirements of the ethics committee. Written informed consent was signed by each subject that enrolled in this study. Our study enrolled 17 subjects with FAP, diagnosed and treated at Tianjin Union Medical Center between January 2010 and June 2015. The diagnostic criteria for FAP patients were as follows: (1) patients having >100 colorectal adenomas or polyps; (2) at least 20 synchronous colorectal adenomas or polyps in patients with a positive family history of FAP. Among these FAP patients, 11 developed cancer, and five of them were randomly selected (group 3) for follow-up study. Of the remaining six cases, three only had small polyps (group 1), and another three had both large and small polyps (group 2). We collected the polyp, cancer, and normal tissues during the operation.

2.2 | Real-time quantitative PCR

Total RNA was extracted from tissue sample using HiPure Fibrous RNA Kit (Magen) based on the manufacturer's protocol. The total RNA purity was determined by $OD_{260/280}$, and its completeness was confirmed by 1.5% of agarose gel electrophoresis. Reverse transcription was accomplished in the 5X All-In-One RT MasterMix (ABM). The qPCR amplifications were performed with an EVAGreen 2X qPCR MasterMix-No Dye Kit (ABM). Each experiment had at least three biological replicates. All the primers were designed and synthesized by Takara Biomedical Technology Co., Ltd. and listed in Table 1. β-actin was as inner control. The relative gene expression levels were calculated using the comparative Ct (ΔΔCt) method, where the relative expression is

TABLE 1 The sequence of primers

Gene	Primer	
KI67	Forward	5′-AGAGTAACGCGGAGTGTCAAGAG-3′
	Reverse	5′-AATATCTTCACTGTCCCTATGACTTCTG-3′
CD31	Forward	5′-AAGTTCAAGTGTCCTCAGCTGAGTCT-3′
	Reverse	5′-GCCTTGCTGTCTAAGTTCCATCA-3′
VEGFA	Forward	5′-ATCGAGTACATCTTCAAGCCAT-3′
	Reverse	5′-ACACGCTCCAGGACTTATACCG-3′
CDH5	Forward	5′-CACTCATGACTGCATGACGGA-3′
	Reverse	5′-GCCTTCTGCAAGGTGTGCCT-3′
β-actin	Forward	5′-CTGGACTTCGAGCAAGAGAT-3′
	Reverse	5′-GATGTCCACGTCACACTTCA-3′

calculated as $2^{-\Delta\Delta Ct}$. The calculation formula is as follows (Mughal et al., 2018):

$$\Delta\Delta Ct = \left(Ct_{tt} - Ct_{tc}\right) - \left(Ct_{ct} - Ct_{cc}\right)$$

Whereas Ct represents the threshold cycle, Ct_{tt} and Ct_{tc} represents Ct values of target gene and inner control in experimental group, Ct_{ct} and Ct_{cc} represents Ct values of target gene and inner control in control group.

2.3 | Western blot analysis

The protein expression level of CD31, VEGF, CDH5, and KI67 was analyzed by western blotting. In brief, following the manufacturer's instructions, polyp, cancer, and normal tissues were homogenized in ice-cold lysis buffer (KeyGEN Whole Cell Lysis Assay) to obtain lysates. After centrifugation at 18,800 (xg) for 30 min at low temperature, the supernatant was taken and the protein concentration was determined by BCA method. The lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked by 5% of nonfat milk in Tris-buffered saline with Tween-20 and incubated overnight with primary antibody at 4°C. Then, the membranes were incubated with secondary antibody for 60 min at 37°C and washed with Tween-20. Band signals were detected with an enhanced chemiluminescence system (Thermo Scientific) and exposure to Kodak X-ray film. Tubulin was used as the internal reference protein. The band intensity was analyzed using Image J software (version 1.8.0). The experiment was repeated three times.

2.4 | Immunohistochemistry

The polyp, cancer, and normal tissues were isolated and performed IHC analysis according to the conventional protocol. In short, all samples were fixed with 10% of formalin and embedded in paraffin, and serially sectioned at 5 μm. The sections were treated with conventional dewaxing. Endogenous peroxidase was blocked with 3% of hydrogen peroxide for 10 min, rinsed with PBS, and incubated with blocking solution for 15 min. The sections were incubated with a monoclonal mouse anti-CD31, anti-VEGF, anti-CDH5, and anti-KI67 antibody at room temperature for 1 h. After washing with PBS, the sections were incubated with secondary antibody for 30 min at 37°C. Diaminobenzidine (DAB) coloration kit was used to stain the slides according to the instructions. Then, it was counterstained with hematoxylin, and sealed after routine dehydration. Photographs were observed under an optical microscope. Each experiment was repeated three times.

2.5 | Statistical analysis

The results are expressed as mean ± standard deviation. SPSS statistical software 21.0 was used to process data. One-way ANOVA or independent sample *t*-test was conducted for comparison among different types of tissues. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | The expression of cell proliferation marker KI67

The expression level of *KI67* mRNA in polyp tissue was significantly higher than that in normal tissue ($p < 0.05$, Figure 1a). Compared with normal tissue and large polyps tissue, *KI67* mRNA expression in small polyp was prominently lower ($p < 0.05$, Figure 2a), while there was not significantly different between large polyp and normal tissue ($p > 0.05$, Figure 2a). *KI67* mRNA expression level in cancer tissue was markedly higher than that in normal and polyp tissues ($p < 0.05$, Figure 3a), and remarkably lower in polyp tissue

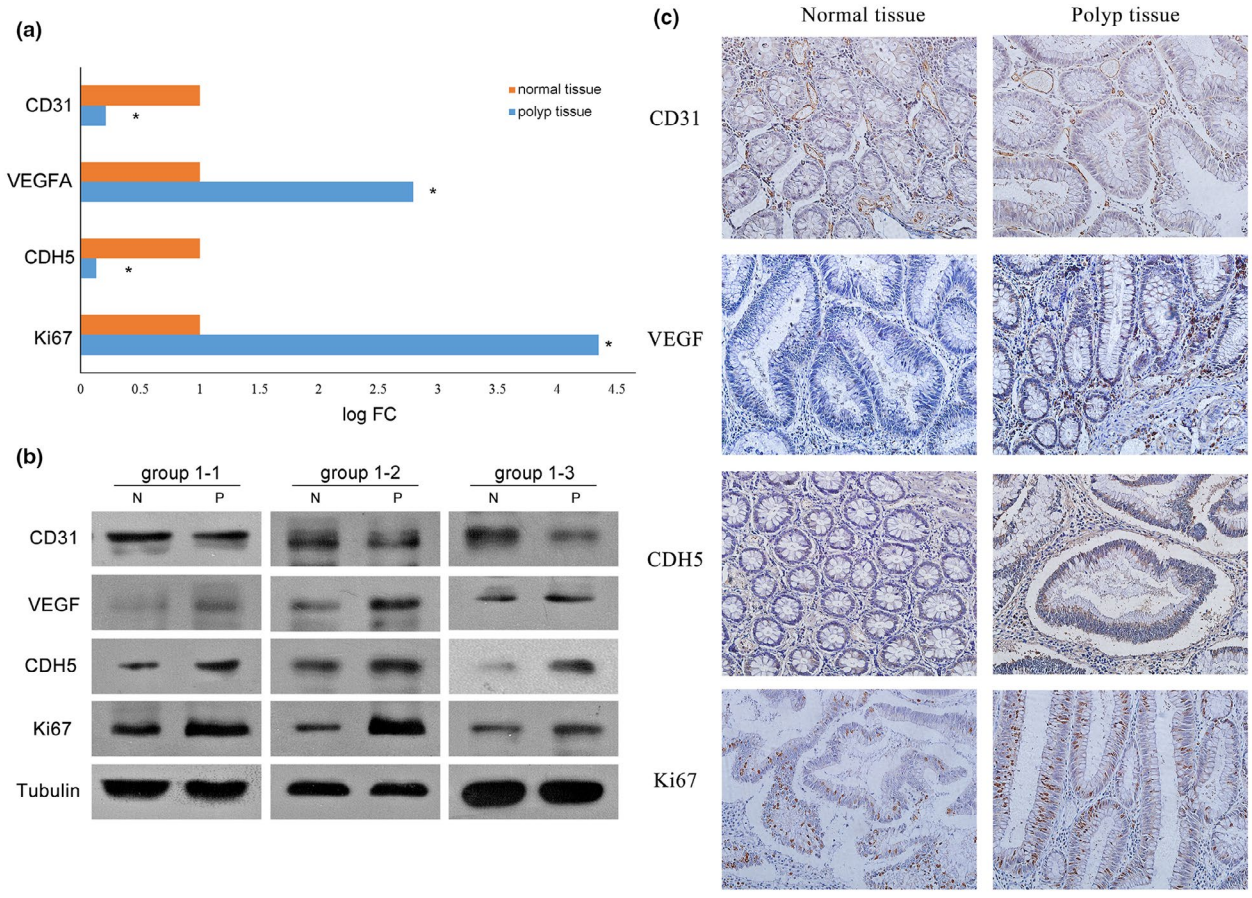

FIGURE 1 The results of qPCR (a), western blot (b), and immunohistochemistry (c) in patients with only small polyp tissue (group 1). Group 1-1 to 1-3 represent case 1 to case 3 in group 1, N represents normal tissue; P represents polyp tissue. *Represents $p < 0.05$ compared with normal tissue

compared with normal tissue ($p < 0.05$, Figure 3a). At the protein level, KI67 expression was increased in polyp tissue compared to normal tissue (Figure 1b,c, Table 2). KI67 protein expression in large polyp tissue was higher than that in normal and small polyp tissues (Figure 2b,c, Table 3). Compared with normal and polyp tissues, the expression of KI67 protein universally increased in cancer tissue (Figure 3b,c, Table 4).

3.2 | The expression of vascular endothelial markers

The *VEGFA* mRNA expression in polyp tissue was notably higher than that in normal tissue ($p < 0.05$, Figure 1a). There were observable difference in the expression of *CD31* and *CDH5* mRNA in normal and polyp tissues, and the expression level was lower in polyp tissue ($p < 0.05$, Figure 1a). Compared with normal and small polyp tissues, the expression of *CD31*, *VEGFA*, and *CDH5* mRNA increased memorably in large polyp tissue ($p < 0.05$, Figure 2a). The expression levels of *VEGFA* and *CDH5* were decreased in polyp and cancer tissues and were remarkably different from normal tissue ($p < 0.05$, Figure 3a). At the protein level, the expression of VEGF and CDH5 were increased in polyp tissue compared to normal tissue (Figure 1b,c, Table 2). The expression of CD31 decreased with the occurrence and enlargement of polyps, while the expression of VEGF and CDH5 increased (Figure 2b,c, Table 3). Compared with normal and polyp tissue, the expression of CD31 was decreased in cancer tissue, while the expression of VEGF and CDH5 was increased (Figure 3b,c, Table 4).

4 | DISCUSSION

How FAP transforms into colorectal cancer (CRC) is a considerable research topic. The abnormal expressions of a large number of genes and proteins, especially those correlated with cell proliferation and angiogenesis, play important roles in this process. In colon cancer, the expression of KI67 indicated the development of lymph node metastasis, significant treatment response, and prognostic value (Fluge et al., 2009; Guzińska-Ustymowicz et al., 2009; Wang et al., 2018).

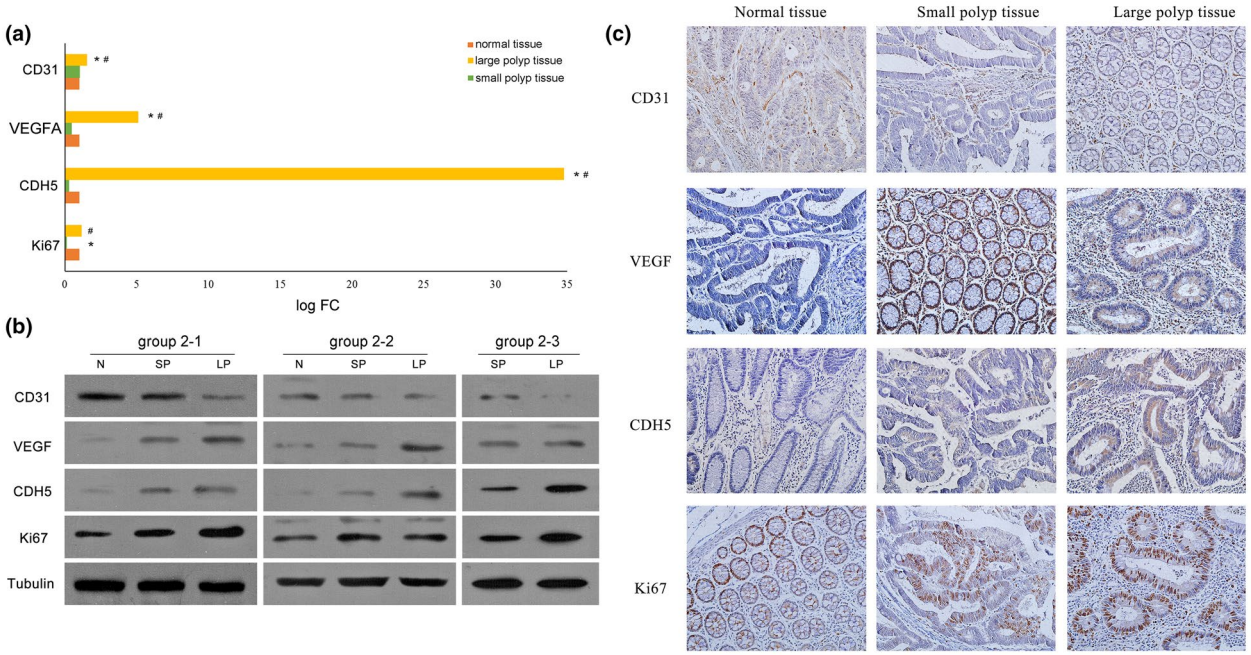

FIGURE 2 The result of qPCR (a), western blot (b), and immunohistochemistry (c) in patients with both large and small polyp tissues (group 2). Group 2-1 to 2-3 represent case 1 to case 3 in group 2, N represents normal tissue, SP represents small polyp tissue, and LP represents large polyp tissue. *Represents $p < 0.05$ compared with normal tissue, and #represents $p < 0.05$ compared with small polyp tissue

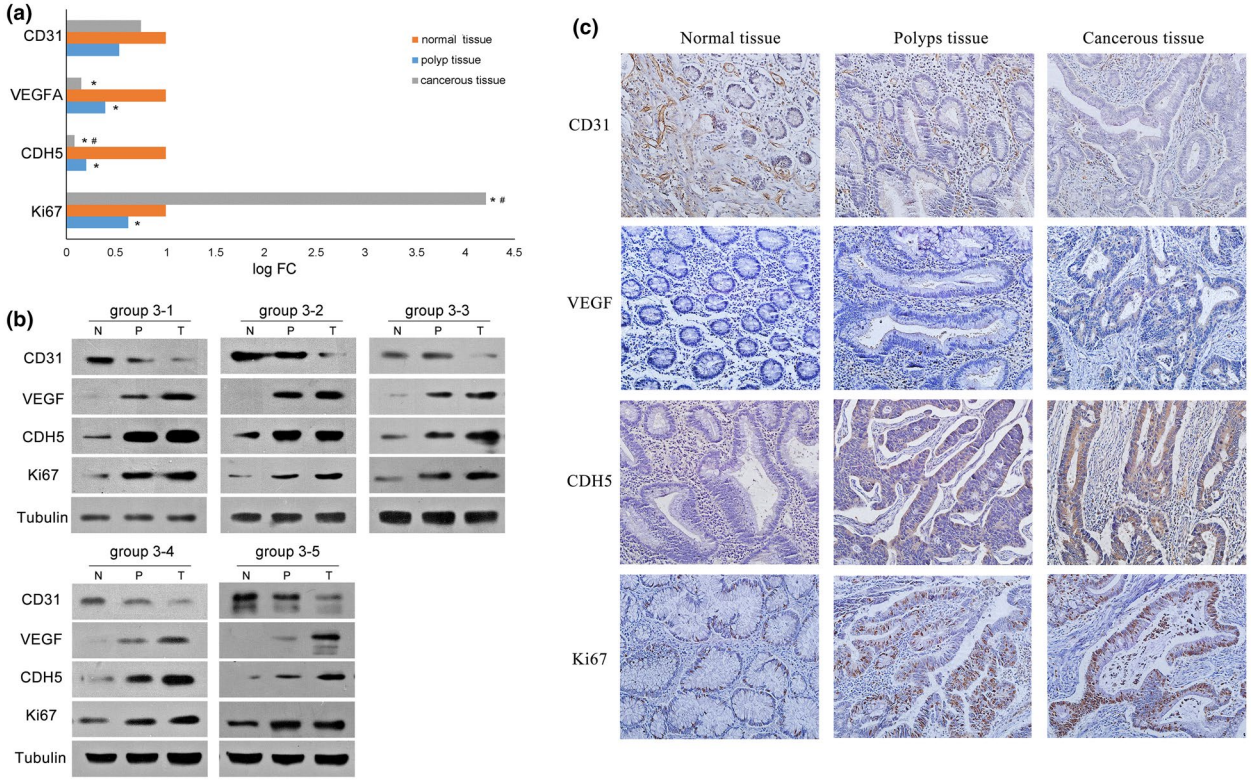

FIGURE 3 The result of qPCR (a), western blot (b), and immunohistochemistry (c) in patients developed cancer (group 3). Group 3-1 to 3-5 represent case 1 to case 5 in group 3, N represents normal tissue, P represents polyp tissue, and T represents cancerous tissue. *Represents $p < 0.05$ compared with normal tissue, and #represents $p < 0.05$ compared with polyp tissue

TABLE 2 The band intensity of CD31, VEGF, CDH5, and KI67 protein (normalized to Tubulin) measured by Image J in paired polyp tissue and normal tissue

	Group 1-1		Group 1-2		Group 1-3	
	N	P	N	P	N	P
CD31	0.92	0.95	0.51	0.48	0.74	0.44
VEGF	0.17	0.32	0.42	0.63	1.05	1.04
CDH5	0.77	0.96	0.59	0.64	0.47	0.95
KI67	0.78	0.95	0.72	0.87	0.89	0.79

Note: N represents normal tissue; P represents polyp tissue.

The related studies have demonstrated that the expression of KI67 in pT3, G2 of CRC may indicate the occurrence of lymph node metastasis (Guzińska-Ustymowicz et al., 2009). Previous studies focused on the expression of KI67 during the development of CRC. In contrast, our results further measured the mRNA and protein expression level of *KI67* in cancer and polyp tissues. In the noncancerous patients, *KI67* mRNA expression was significantly different in small polyps compared with normal tissue or large polyps. In patients with cancer, the expression level of *KI67* mRNA in cancer tissue was significantly higher than that in normal and polyp tissues. At the protein level, KI67 protein expression increased in cancer tissues compared with normal and polyp tissues. Therefore, KI67 can be used as a marker for the carcinogenesis of FAP adenoma.

Vascular growth factor secreted by tumor cells and endothelial cells can stimulate tumor angiogenesis and the growth of tumor cells (Li et al., 2018). *VEGFA*, the most effective of these cytokines, can directly stimulate the migration, proliferation, and division of vascular endothelial cells and accelerate microvascular permeability (Siveen et al., 2017). The VEGF(A) expression was significantly correlated with advanced stage and metastases, it may play an important role in the invasion and metastasis of CRC (Beştaş et al., 2014; Martins et al., 2013), and can be used as prognostic molecular biomarker for CRC patients with liver metastasis (Goos et al., 2016). *CDH5* is an endothelial cell marker and contributes to vasculogenic mimicry (Mao et al., 2013). *CDH5* can regulate VEGF function, and Zanetta et al. forcefully investigated that downregulation of *CDH5* may have significant influence on the growth and bleeding complications of endothelial tumors (Zanetta et al., 2005). However, the expression of *VEGFA* and *CDH5* have not been reported in

TABLE 3 The band intensity of CD31, VEGF, CDH5, and KI67 protein (normalized to Tubulin) measured by Image J in paired large polyp tissue, small polyp tissue, and normal tissue

	Group 2-1			Group 2-2			Group 2-3	
	N	SP	LP	N	SP	LP	SP	LP
CD31	1.03	0.79	0.40	0.56	0.47	0.36	0.67	0.17
VEGF	0.18	0.43	0.65	0.32	0.33	0.63	0.38	0.54
CDH5	0.18	0.47	0.40	0.19	0.31	0.61	0.92	1.02
KI67	1.00	1.07	1.08	0.76	0.99	0.90	1.06	0.98

Note: N represents normal tissue, SP represents small polyp tissue, and LP represents large polyp tissue.

TABLE 4 The band intensity of CD31, VEGF, CDH5, and KI67 protein (normalized to Tubulin) measured by Image J in paired polyp tissue, cancerous, and normal tissues

	Group 3-1			Group 3-2			Group 3-3		
	N	P	T	N	P	T	N	P	T
CD31	1.56	1.46	0.59	1.38	1.33	0.73	0.69	0.74	0.50
VEGF	0.34	1.35	1.47	0.17	1.25	1.30	0.25	1.02	1.09
CDH5	1.21	1.70	1.46	1.32	1.32	1.31	0.70	0.98	1.13
KI67	1.09	1.78	1.56	0.53	1.15	1.32	0.43	0.82	1.03

	Group 3-4			Group 3-5		
	N	P	T	N	P	T
CD31	0.62	0.46	0.46	0.61	0.58	0.38
VEGF	0.11	0.42	0.78	0.05	0.21	0.78
CDH5	0.37	0.94	1.01	0.25	0.63	0.92
KI67	0.67	0.94	1.04	0.72	0.82	0.82

Note: N represents normal tissue, P represents polyp tissue, and T represents cancerous tissue.

the process of adenoma to carcinogenesis in FAP patients. In the current study, the *VEGFA* mRNA and *CDH5* mRNA expression were observably higher in large polyp tissues than that in both small polyp and normal tissues in the noncancerous patients. In cancerous patients, the *VEGFA* mRNA and *CDH5* mRNA expression were dramatically lower in both polyp and cancer tissues than those in normal tissues, but the protein levels of higher in cancer samples than in normal tissues and polyp tissues, recommended that VEGF and CDH5 might play a role in the process of adenoma to carcinogenesis in FAP patients at the protein level, but not at the gene level.

CD31, also known as platelet endothelial adhesion molecule (PECAM-1), has been implicated in the late progression of metastatic tumors. Kuang et al. showed that the expression of CD31 protein could be a potential prognostic factor and therapeutic target in non-small cell lung carcinoma (NSCLC) (Kuang et al., 2013). Furthermore, another research demonstrated that high expression of CD31 was associated significantly with better survival and might be as prognostic factor for renal cell cancer (Virman et al., 2015). However, CD31 is less studied in colon cancer. In present study, the level of *CD31* gene in large polyp tissues was meaningfully higher than that in normal and small polyp tissues in the noncancerous patients. But, in the cancerous patients, the *CD31* mRNA expression was not significantly different in normal, polyp, and cancer tissues. Moreover, the expression of CD31 protein in cancer tissues was lower than that in normal tissues and polyp tissues. It indicated that, similar to VEGF, CD31 might play a role in the process of canceration of FAP at the gene level, not at the protein level.

In summary, our study investigated the mRNA and protein levels of markers associated with tumor proliferation and angiogenesis in normal samples, large and small polyp tissue, and cancer samples. The results suggested that abnormal expression of CD31, KI67, VEGF, and CDH5 might be correlated with the process of adenoma to carcinogenesis in FAP. Nevertheless, further research is needed to clarify the related mechanism.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

XZ designed the study. ZZ and DW performed the experiments, XZ, CX, YL, YY, CC, and ML analyzed the data. XZ, ZZ, and DW wrote the manuscript. All authors reviewed the manuscript and approved the final version.

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