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Case Control Study

Expression and significance of pigment epithelium-derived factor and vascular endothelial growth factor in colorectal adenoma and cancer

Yang Y *et al*. PEDF and VEGF in CRC

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Abstract

BACKGROUND

The incidence and mortality of colorectal cancer (CRC) are among the highest in the world, and its occurrence and development are closely related to tumor neovascularization. When the balance between pigment epithelium-derived factors (PEDF) that inhibit angiogenesis and vascular endothelial growth factors (VEGF) that stimulate angiogenesis is broken, angiogenesis is out of control, resulting in tumor development. Therefore, it is very necessary to find more therapeutic targets for CRC for early intervention and later treatment.

AIM

To investigate the expression and significance of PEDF, VEGF, and CD31-stained microvessel density values (CD31-MVD) in normal colorectal mucosa, adenoma, and CRC.

METHODS

In this case-control study, we collected archived wax blocks of specimens from the Digestive Endoscopy Center and the General Surgery Department of Chengdu Second People's Hospital from April 2022 to October 2022. Fifty cases of specimen wax blocks were selected as normal intestinal mucosa confirmed by electronic colonoscopy and concurrent biopsy (normal control group), 50 cases of specimen wax blocks were selected as colorectal adenoma confirmed by electronic colonoscopy and pathological biopsy (adenoma group), and 50 cases of specimen wax blocks were selected as CRC confirmed by postoperative pathological biopsy after inpatient operation of general surgery (CRC group). An immunohistochemical staining experiment was carried out to detect PEDF and VEGF expression in three groups of specimens, analyze their differences, study the relationship between the two and clinicopathological factors in CRC group, record CD31-MVD in the three groups, and analyze the correlation of PEDF, VEGF, and CD31-MVD in the colorectal adenoma group and the CRC group. The *F* test or adjusted *F* test is used to analyze measurement data statistically. Kruskal-Wallis rank sum test was used between groups for ranked data. The chi-square test, adjusted chi-square test, or Fisher's exact test were used to compare the rates between groups. All differences between groups were compared using the Bonferroni method for multiple comparisons. Spearman correlation analysis was used to test the correlation of the data. The test level (a) was 0.05, and a two-sided $P< 0.05$ was considered statistically significant.

RESULTS

The positive expression rate and expression intensity of PEDF were gradually decreased in the normal control group, adenoma group, and CRC group (100% *vs* 78% *vs* 50%, $c^2 = 34.430$, $P < 0.001$; ++~++ *vs* +~++ *vs* -~+, $H = 94.059$, $P < 0.001$), while VEGF increased gradually (0% *vs* 68% *vs* 96%, $c^2 = 98.35$, $P < 0.001$; - *vs* -~+ *vs* ++~+++, $H = 107.734$, $P < 0.001$). In the CRC group, the positive expression rate of PEDF decreased with the increase of differentiation degree, invasion depth, lymph node metastasis, distant metastasis, and TNM stage ($c^2 = 20.513, 4.160, 5.128, 6.349, 5.128$, $P < 0.05$); the

high expression rate of VEGF was the opposite (c^2 = 10.317, 13.134, 17.643, 21.844, 17.643, P < 0.05). In the colorectal adenoma group, the expression intensity of PEDF correlated negatively with CD31-MVD (r = -0.601, P < 0.001), whereas VEGF was not significantly different (r = 0.258, P = 0.07). In the CRC group, the expression intensity of PEDF correlated negatively with the expression intensity of CD31-MVD and VEGF (r = -0.297, P < 0.05; r = *-0.548*, P < *0.05),* while VEGF expression intensity was positively related to CD31-MVD (r = 0.421, P = 0.002).

CONCLUSION

It is possible that PEDF can be used as a new treatment and prevention target for CRC by upregulating the expression of PEDF while inhibiting the expression of VEGF.

Key Words: Pigment epithelium-derived factors; Vascular endothelial growth factor; Microvessel density; Colorectal adenoma; Colorectal cancer; Targeted therapy

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Core Tip: Targeted therapy is one of the most widely recognized and accepted methods for the treatment of colorectal cancer (CRC). Recent years have brought an intense focus to the study of the angiogenic signaling pathway, there have been studies showing that the infiltration, staging, and metastasis of colorectal are related to pigment epithelium-derived factors (PEDF) and vascular endothelial growth factors, but the current domestic and foreign studies on PEDF almost do not involve colorectal adenoma, a precancerous lesion. In our study, colorectal adenoma, a precancerous lesion, was added to analyze and explore the possibility of PEDF as a new target for early prevention and later treatment of CRC.

INTRODUCTION

Colorectal cancer (CRC) has become the third most prevalent cancer, and its mortality rate ranks second in the world[1], every year, approximately 1 million new cases are diagnosed[2]. According to estimates, 3.2 million new cases of CRC will be diagnosed by 2040, while 1.6 million people will die from the disease[3]. Therefore, human health has been seriously threatened by CRC. The evolution of the sequence of "normal intestinal epithelium → adenoma → cancer" represents the occurrence process of most CRCs[4,5]. Colorectal adenoma is a major precancerous disease of CRC, accounting for at least 70%-90% of all precancerous diseases of CRC[6,7]. Endoscopic resection of adenomas is recognized as an effective method to prevent CRC, but the recurrence rate of adenomas in situ and at other sites after resection is still high[8,9], and subsequent chemoprevention, regular follow-up colonoscopy, and even repeated resection are still required. In recent years, advances in the understanding of species biology have facilitated the development of targeted therapies and also provided new ideas for the treatment of CRC. Finding a new target that can prevent colorectal adenoma from progressing to CRC and treat CRC at the same time deserves further study.

In the development of any solid tumor, the growth of neovascularization is essential[10]. CRC is one of the many malignant solid tumors involving angiogenesis, and angiogenesis is also crucial in the occurrence and development of CRC. Although vascular endothelial cells are normally quiescent, proangiogenic factors such as vascular endothelial growth factor (VEGF) can induce sprouting and initiate the formation of new blood vessels[11]. VEGF is a highly specific mitogen that plays an important role in angiogenesis and neovascularization. It was independently isolated and discovered in 1989 by Leung *et al*[12], and in 1993, it was found that inhibition of VEGF-induced angiogenesis with specific monoclonal antibodies significantly inhibited the growth of a variety of tumors[13]. These findings provided important evidence that inhibition of angiogenesis can suppress growth and lead to tumor blocking. At present, the research on VEGF in CRC has been relatively mature. Existing studies have

confirmed that the expression of VEGF is up-regulated in CRC, and it is negatively correlated with tumor stage, metastasis and prognosis[14,15]. VEGF level increases with the increase of CRC stage, and it can be used as an independent predictor of overall survival in patients with CRC[16]. The prognosis of CRC patients with high expression of VEGF is poor[17]. Angiogenesis is crucial for tumor growth and metastasis. The expression of VEGF is related to the increase of microvessel density in CRC. VEGF is not only a major mediator of angiogenesis, but also a key factor promoting the formation of vascular endothelial cells and lymphatic vessels[18]. It promotes the occurrence and development of CRC and is an important angiogenic factor in primary and metastatic CRC. In addition, some researchers have done relevant studies on whether VEGF can predict the pathological complete response (pCR) of preoperative chemoradiotherapy (preCRT) in rectal cancer, and the results show that patients with high expression of VEGF have a significantly higher pCR rate[19]. VEGF can not only be used as a prognostic factor for CRC, but also can be used to predict the response to conventional systemic therapy and local radiotherapy in CRC. During the recent years, as scholars have studied the angiogenesis signaling pathway in-depth, it has been found that neovascularization is already active in the earliest stage of CRC occurrence and development[20]. In colorectal adenomas, some studies have found that VEGF is highly expressed in colorectal adenomas and low-grade intraepithelial neoplasia[15], and VEGF may be used to risk stratifying intestinal polyps with different risk of progression[21]. However, few studies have investigated the relationship between VEGF expression and angiogenesis in the sequence of "normal intestinal epithelium → adenoma → carcinoma" from the early stage of CRC.

In the process of CRC occurrence and progression, the balance between stimulating and inhibiting factors of angiogenesis is destroyed, which causes CRC to develop abnormal blood vessels, and further promotes the occurrence and progression of tumors. As the core factors regulating tumor vascular microenvironment, pigment epithelium-derived factors (PEDF) play a key role in regulating tumor angiogenesis and blood supply metastasis[22]. The ratio of PEDF/VEGF finely regulates blood vessel

formation, and the balance between the two plays a crucial role in angiogenesis[23,24]. PEDF is an endogenous neovascularization inhibitor, also known as early population double level c DNA-1 (EPC-1), which is composed of 418 amino acids with a molecular mass of about 50 kDa. PEDF was first identified as a neuronal differentiation inducer in conditioned medium of human retinal pigment epithelium cells in 1989. And it is an inducer of neuronal differentiation in Y79 retinoblastoma cells[25]. PEDF shares structural and sequence homology with members of the serine protease inhibitor (SERPIN) superfamily[26]. In 1999 Dawson *et al*[27] found that PEDF has potent antiangiogenic activity and is more potent than angiostatin in inhibiting angiogenesis. Significant reductions in PEDF levels have been found in agerelated macular degeneration and diabetic retinopathy, two pathological processes dependent on angiogenesis[28,29]. PEDF can also induce the differentiation of neuroblastoma tumor cells and promote the neuroendocrine function of prostate cancer cells[30,31]. PEDF, encoded by the SERPINF1 gene, first appeared in vertebrates and has shown strong conservation in the evolution of mammalian species. The PEDF gene is widely expressed in eye, prostate, mammary gland, cervix, lung, pancreas, liver, colorectal and other tissues, and the regulatory and biological role of the gene is preserved in spinal animals[32]. The human PEDF gene is located in 17p13.1, which is a region containing a group of cancer-related genes[33,34]. This also indicates that PEDF, as a multifunctional protein, not only participates in physiological and pathological reactions such as neuroprotection, regulation of oxidative stress, inhibition of blood vessels, osteogenesis, anti-inflammation, lipid metabolism [35-38], but also may have anti-tumor effects.

Compared with normal tissues, the expression of PEDF is decreased in cancer tissues of solid tumors such as gonadal tumors, lung cancer, and pancreatic cancer[39-42], suggesting that the loss of PEDF may play a key role in tumorigenesis. Gene therapy of PEDF or PEDF therapy with recombinant proteins has been used in ovarian cancer and lung cancer[43,44]. PEDF can play an anti-tumor role by inhibiting tumor angiogenesis, proliferation, migration, invasion and metastasis of tumor cells, and inducing apoptosis of cancer cells[45]. The expression of PEDF is closely related to tumor progression and

survival prognosis, and the low expression of PEDF often predicts tumor progression and shorter survival time[46]. Among the few studies on PEDF in CRC at home and abroad, most of them showed that the expression level of PEDF in CRC tissues was lower than that in adjacent tissues, and its expression was negatively correlated with tumor stage[47]. However, some studies showed that the expression of PEDF in CRC tissues was not significantly different from that in paired normal tissues[48]. In addition, the current research on PEDF at home and abroad almost does not involve colorectal adenoma, which is a precancerous disease.

At present, endothelial cells are attractive targets for the treatment of diseases that depend on the formation of new blood vessels, such as cancer. The activity of neovascularization has occurred in the earliest stage of CRC, and the formation of blood vessels runs through the entire occurrence and development process of CRC[47]. VEGF-targeted drugs are effective and safe for treating CRC have been confirmed and widely promoted[49]. PEDF related formulation mainly include peptide formulation, physical and chemical carriers, and biological carriers. At present, no toxicity caused by PEDF formulation itself has been observed in anti-tumor vascular animal models. Therefore, we boldly speculate that PEDF, as an antagonist of VEGF, may become a new target for early prevention and later treatment of CRC.

Microvessel density (MVD) has been regarded as an extremely important marker of tumor microangiogenesis by researchers. CD31 is selected by immunohistochemical staining to mark microvessels, and microvessel density is calculated, which is a commonly used detection method for quantitative analysis of tumor angiogenesis[50]. In this study, we investigated the expression of PEDF and VEGF in normal colorectal mucosa, adenomas and CRC, and their relationship with the clinicopathological characteristics of CRC, starting from the earliest stage of CRC development and including colorectal adenoma, a precancerous lesion. At the same time, the microvessels were marked with CD31, and the MVD of each tissue was calculated, and the difference and correlation between them were analyzed. To investigate the role and significance of

PEDF and VEGF in the pathogenesis of CRC from normal intestinal epithelium to adenoma and then to cancer.

MATERIALS AND METHODS

Materials

We collected the archived wax blocks of specimens submitted by the Department of Digestive Endoscopy Center and General Surgery of Chengdu Second People's Hospital from April 2022 to October 2022. Fifty cases of specimen wax blocks were selected as normal intestinal mucosa confirmed by electronic colonoscopy and concurrent biopsy (normal control group), 50 cases of specimen wax blocks confirmed as colorectal adenoma by electronic colonoscopy and pathological biopsy (colorectal adenoma group), and 50 cases of specimen wax blocks were selected as CRC confirmed by postoperative pathological biopsy after inpatient operation in the Department of General Surgery (CRC group).

Inclusion criteria: (1) Pathological biopsy confirmed that all specimens were normal colorectal mucosa, colorectal adenoma, or CRC, respectively; (2) The included specimens had complete case data; and (3) None of the patients with CRC included had a history of colorectal surgery, and none had received chemoradiotherapy or other anti-tumor therapy.

Exclusion criteria: (1) patients previously diagnosed with other malignant tumors; (2) patients with eye and immune system diseases; and (3) patients with a combined history of intestinal tuberculosis, familial intestinal polyposis, inflammatory bowel disease, and hamartomatous polyposis syndrome.

This study was approved by Chengdu Second People's Hospital's Ethics Committee and all patients signed informed consent forms.

Immunohistochemistry

We used a rotary microtome (Leica, Germany) to re-cut each of the above-selected wax blocks into 3 consecutive slices with a thickness of 3um and used the Roche BenchMark

GX automatic immunohistochemical dye machine for immunohistochemical staining of PEDF, VEGF, and CD31. Rabbit anti-human VEGF monoclonal antibody (UK Abcam) working concentration: 1:100; rabbit anti-human CD31 polyclonal antibody (UK Abcam) working concentration: 1:2000; rabbit anti-human PEDF polyclonal antibody (US GeneTex) working concentration: 1:500; PBS phosphate buffer (Fuzhou Maixin Technology Development Co., LTD., China); DAB dyeing solution (Ventana Medical Systems, United States).

Every batch of experiments was accompanied by positive and negative controls. Negative controls were PBS buffer rather than primary antibodies, and the positive control was referred to as a known positive image.

Automatic immunohistochemical dyeing machine dyeing process: (1) Baking: temperature 75 °C, time 4 min; (2) Dewaxing: add EZ prep liquid, dewaxing temperature 76 °C for 4 min, then rinse the sections with EZ prep liquid twice; (3) Antigen repair: hot repair temperature of 99 °C, incubation time of 30 min, repair solution: CC1, PH 8.5, rinse with reaction buffer after repair; (4) Block endogenous peroxidase: add 100 μL inhibitor, add oil membrane LCS, incubate at 37 °C for 4 min, and rinse reaction buffer after incubation; (5) Incubate for 32 min at 37 °C with the primary antibody. Rinse with reaction buffer after incubation; (6) The second antibody was incubated at 37 °C for 8 min and rinsed with reaction buffer after incubation; (7) Color development: Add 100 μL of DAB and 100 microliters of H_2O_2 for color development, then add oil film LCS, incubate at 37 °C for 8 min, and rinse with reaction buffer after incubation; (8) Add 100 μL of color enhancer, incubate at 37 °C for 4 min, and rinse with reaction buffer after incubation; (9) Interlining: Add 100 μL hematoxylin II, incubate at 37 °C for 8 min, and rinse with reaction buffer after incubation; (10) Interlining: blue return, temperature 37 °C, incubation time 4 min, rinse with reaction buffer after incubation; and (11) Finish dyeing.

Interpretive standard

Immunohistochemical interpretation criteria for PEDF and VEGF were as follows: PEDF positive expression was located in the nucleus, and study cells with light yellow, yellow, or brownish-yellow nuclei in tissue sections were identified as positive cells. VEGF is widely expressed in large intestine stromal cells and vascular endothelial cells in yellow or brownish yellow color, and this expression is used as a positive internal control in the interpretation of VEGF, and the study cells with light yellow, yellow, or brownish-yellow cytoplasm in the tissue section are judged as positive cells. First, the whole film was scanned with a low-power lens (100 ×) to preliminatively determine whether there were positive cells. Then, the study cell distribution area was switched to a high-power lens (200 ×) to observe 5 visual fields, and a comprehensive score was scored on the strength of staining and the number and percentage of positive cells. A five-grade system was used to score the percentage of positive cells: zero points for no positive cells, one point for 1%-25%, two points for 25%-50%, three points for 50%-75%, and four points for over 75%. The dyeing intensity score is divided into four levels: 0, 1, 2, and 3 points for no staining, light yellow, yellow, and brownish-yellow. The final score of the staining result = (percentage score of the number of positive cells above) × (staining intensity score); the final score of 0 is judged as negative (-), 1-4 is judged as weak positive (+), 5-8 is judged as medium positive (+), and 9-12 is judged as strong positive (+++). Counting microvessels marked by CD31 staining: Brown-colored endothelial cells and clusters can be counted as microvessels if they are separated from adjacent blood vessels, tumor cells, and other connective tissues. The entire film is first scanned with a low-power lens (100 ×) in order to find areas where the microvascular density is evenly distributed, so as to identify areas of high-density blood vessels, which are called "hot spots". Then each section was observed in 5 random fields of the "hot spot" area under a high-power lens (200 ×), and a mean microvascular density value was determined by the average number of blood vessels in each field.

The above results were interpreted by two experienced film readers in the department of pathology who independently read the tissue sections in double-blind

conditions. If the score of the film reading results for the same tissue section was inconsistent, the average score used was taken as final.

Statistical analysis

The statistical review of the study was conducted by a biomedical statistician. All data were analyzed and processed by IBM SPSS Statistics 26.0 (Armonk, NY, United States). The measurement data in this experimental study followed the normal distribution and were statistically described in the form of mean ± SD. The *F*-test or corrected *F*-test (Welch's test) was used for statistical analysis of measurement data. The Kruskal-Wallis rank sum test was used between groups for ranked data, and if statistical differences between groups existed, the Bonferroni method was further used for multiple comparisons. The counting data were described in the form of the number of cases (percentage). Chi-square test, corrected chi-square test or Fisher's exact test were used to complete the comparison of rates between groups and if the difference between groups was statistically significant, Bonferroni method was further used for pairwise comparison. Spearman correlation analysis was used to test the correlation of the data. The test level (α) was 0.05, and a two-sided $P < 0.05$ was considered statistically significant.

RESULTS

General data

In the normal control group, there were 25 cases (50%) of males and 25 cases (50%) of females, aged 24-80 years old, with an average age of 55.58 ± 13.670 years old. Pathological specimens were obtained from the rectum in 15 cases (30%), the left half colon in 23 cases (46%), and the right half colon in 12 cases (24%). In the colorectal adenoma group, there were 28 males (56%) and 22 females (44%), aged 31-84 years, with an average age of 56.46 ± 12.755 years. Pathological specimens were obtained from the rectum in 14 cases (28%), the left colon in 23 cases (46%), and the right colon in 13 cases (26%). In the CRC group, there were 33 males (66%) and 17 females (34%), aged

30-91 years, with an average age of (60.16 ± 14.435) years. Pathological specimens were obtained from the rectum in 24 cases (48%), the left half colon in 14 cases (28%), and the right half colon in 12 cases (24%). The three groups did not differ statistically significantly in gender(c^2 = 2.617, P = 0.263) (Figure 1 A), age (F = 1.588, P = 0.208) (Figure 1 B), or specimen source location (c^2 = 6.188, P = 0.186) (Figure 1 C).

Positive expression of PEDF and VEGF

Normal control subjects had the highest positive expression rate of PEDF, followed by the colorectal adenoma group, and the CRC group had the lowest (100%, 78%, 50%). However, positive expression rates for VEGF were highest among CRC group, followed by colorectal adenoma group, and lowest among normal control subjects (96%, 68%, 0%). Positive expression rates for PEDF and VEGF were significantly different among all groups (P < 0.05) (Figure 2 A and C).

Expression intensity of PEDF and VEGF

There were statistically significant differences between the three groups in terms of PEDF and VEGF expression intensity (P < 0.001), and there were statistically significant differences in the expression intensity of PEDF and VEGF in the three groups, respectively (P < 0.001) (Figure 2 B and D). The expression intensity of PEDF was the highest in the normal control group, with mainly medium positive (++) and strong positive (+++) expression (Figure 3 A and B). And in the colorectal adenoma group, weak positive (+) and medium positive (++) were predominant (Figure 3 C and D). The lowest was found in the CRC group, with negative (-) and weakly positive (+) expressions predominant (Figure 3 E and F). On the contrary, the expression intensity of VEGF was the highest in the CRC group, with mainly medium positive (++) and strong positive (+++) expression (Figure 4 E and F). In the colorectal adenoma group, the expression of negative (-) and weak positive (+) was the second (Figure 4 C and D). The lowest was found in the normal control group, all of which had negative (-) expression (Figure 4 A and B).

VEGF, PEDF, and cancer clinicopathology

Table 1 shows that the positive expression rate of PEDF in CRC was not statistically different in terms of age, gender, tumor size, and tumor location (Figure 5 A). But there were statistical differences in the degree of differentiation, depth of invasion, presence or absence of lymph node metastasis, presence or absence of distant metastasis, and clinical stage ($P < 0.05$) (Figure 5 C). In the CRC group, the positive expression rate of PEDF was higher in highly differentiated cancers than in medium-low differentiated cancers, higher in carcinomas without serosal invasion than in carcinomas with serosal invasion, higher in cancers without lymph node metastasis than in cancers with lymph node metastasis, and higher in cancers without distant metastasis than in cancers with distant metastasis. The positive expression rate of PEDF in AJCC stage I + II cancer was higher than that in stage III + IV cancer.

In the CRC group, the negative expression rate of VEGF was only 4%, but the positive expression rate was as high as 96%, among which the weak positive (+), medium positive (++), and strong positive (+++) expressions accounted for 14%, 52%, and 30%, respectively. Therefore, we classified negative (-), weak positive (+), and moderate positive (++) expression as low expression, and strong positive (+++) expression as high expression. The statistical results showed that the high expression rate of VEGF in CRC had no statistical difference with age, sex, tumor size, or tumor site (Figure 5 B, Table 1). The high expression rate of VEGF in CRC had statistical differences with the degree of differentiation, depth of invasion, presence of lymph node metastasis, presence of distant metastasis, and clinical stage ($P < 0.001$) (Figure 5 D, Table 1). In CRC, the high expression rate of VEGF is higher in medium-low differentiated cancers than in highly differentiated cancers, and higher in carcinomas with serosal invasion than in carcinomas without serosal invasion, higher in cancers with lymph node metastasis than in cancers without lymph node metastasis, higher in cancers with distant metastasis than in cancers without distant metastasis, and higher in cancers with clinical stage III+IV than in cancers with stage I + II.

CD31-MVD

The CD31-MVD values of the normal group were 1.012-1.180/HP, and the average microvascular density of each high-power (200 ×) field was (1.096 ± 0.2948)/HP. In the adenoma group, the CD31-MVD values were 11.683-14.085/HP, and the average microvascular density was 12.884 ± 4.2267)/HP under each high-power (200 ×) field of view. In the CRC group, the CD31-MVD values ranged from 30.507 to 35.253/HP, and the average microvascular density was 32.88 ± 8.3488/HP per high-power (200 ×) field of view. There were statistical differences in CD31-MVD values among the normal group, adenoma group, and CRC group ($P < 0.001$) (Figure 6A). In the CRC group, CD31-MVD values were highest, adenoma values were second, and normal values were lowest (Figure 7).

Correlation between PEDF, VEGF and CD31-MVD

The expression intensity of PEDF was statistically significantly different from CD31-MVD value in the adenoma group ($r = -0.601$, $P < 0.001$) (Figure 6B). There was a negative correlation between PEDF expression intensity and CD31-MVD value, and the CD31-MVD value increased with the decrease in PEDF expression. However, the correlation between VEGF expression intensity and CD31-MVD value was not statistically significant ($r = 0.258$, $P = 0.07$) (Figure 6C).

In the CRC group, the expression intensity of PEDF was negatively correlated with the CD31-MVD value ($r = -0.297$, $P = 0.036$), and the expression intensity of PEDF increased with the decrease in PEDF expression (Figure 6E). The correlation between PEDF expression intensity and VEGF expression intensity was also statistically significant ($r = -0.548$, $P < 0.001$) (Figure 6D). The expression intensity of PEDF was negatively correlated with that of VEGF, and the expression intensity of VEGF increased with the decrease in PEDF expression. In addition, the correlation between VEGF expression intensity and CD31-MVD value was also statistically significant ($r = 0.421$, $P = 0.002$) (Figure 6F). The expression intensity of VEGF was positively correlated

with the CD31-MVD value, and the CD31-MVD value increased with the increase in VEGF expression.

ROC curve

We analyzed the value of PEDF, VEGF, and PEDF + VEGF in diagnosing CRC using a ROC curve. The AUC of PEDF in the diagnosis of CRC was 0.842, the 95% confidence interval was 0.779-0.940, the sensitivity was 86%, the specificity was 74%, and the best cut-off value was weak positive (+) expression. The AUC of VEGF in the diagnosis of CRC was 0.936, the 95% confidence interval was 0.891-0.981, the sensitivity was 82%, the specificity was 96%, and the best cut-off value was moderate positive (++) expression. The AUC of PEDF + VEGF in the diagnosis of CRC was 0.935, the 95% confidence interval was 0.887-0.984, the sensitivity was 82%, and the specificity was 96%. There was a statistically significant difference in AUC when detecting PEDF, VEGF, and PEDF + VEGF in tissues to diagnose CRC ($P < 0.001$) (Figure 8).

DISCUSSION

This experimental study showed that PEDF was expressed in normal colorectal mucosa, colorectal adenoma tissue, and CRC tissue, and the results of our study were in accordance with the findings of Ji *et al*[47]. At the same time, this study also complements the current research on the difference in the expression of PEDF in colorectal adenoma tissues, normal colorectal mucosa, and CRC tissues. In addition, the positive expression rate and intensity of PEDF in normal colorectal mucosa, adenoma, and cancer tissues gradually decreased during the development of CRC, while that of VEGF was the opposite. The positive expression rate of PEDF and the high expression rate of VEGF were found to be related to the degree of differentiation, depth of invasion, lymph node metastasis, and distant metastasis of CRC, as well as the clinical stage. The positive expression rate of PEDF in well-differentiated carcinoma was higher than that in moderate-poorly differentiated carcinoma, in non-serosal invasion carcinoma was higher than that in serosal invasion carcinomas, in carcinoma without lymph node

metastasis was higher than that with lymph node metastasis, in carcinoma without distant metastasis was higher than that with distant metastasis, and in carcinoma at clinical stage I + II was higher than that in stage III + IV carcinoma; however, the high expression rate of VEGF was in contrast. The results of the study are also consistent with that of Harries *et al*[51] and Das *et al*[52]. The results show that PEDF and VEGF are involved in the whole process of the occurrence and development of CRC. The higher the positive expression rate of PEDF and the lower the high expression rate of VEGF, the higher the degree of differentiation, the lower the probability of serosa invasion, the lower the risk of lymph node and distant metastasis, and the better the clinical stage of CRC. Therefore, we can speculate that the expression of PEDF is inhibited in the evolution process of "normal intestinal epithelium → adenoma → cancer", and it plays an inhibitory role in the development process of malignant transformation of colorectal adenomatous polyps and the progression of CRC. PEDF is a protective factor in the occurrence and development of CRC. However, the clinical stage of CRC with high expression of VEGF is poor, and VEGF is a promoting factor in the progression of CRC. Detection of VEGF expression in CRC may provide valuable clinical staging and prognostic information for CRC patients, which is also consistent with the results of earlier meta-analysis[53].

Angiogenesis is a multi-step process triggered by a variety of biological signals, involving the activation, migration, tube formation, differentiation and maturation of vascular endothelial cells[54]. Angiogenesis mainly includes sprouting angiogenesis and intussusceptive angiogenesis. The former grows new capillaries from the previous capillaries and then forms new blood vessels. The latter is a novel mode of vessel formation and remodeling that can lead to the formation of new blood vessels by internal division of preexisting capillary plexus[55]. In addition, angiogenesis is very important in different stages of cancer, and angiogenesis may also be a fundamental step in the transformation of tumors from benign to malignant. Since the early 1990s, MVD has been considered one of the indicators for tumor prognosis research[32]. MVD detection of CRC and MVD detection of precancerous diseases can better explore the

occurrence and development processes of tumors. In this experimental study, there were statistical differences in CD31-MVD values among the three groups. In each group, CRC showed the highest CD31-MVD value, followed by colorectal adenoma, and normal colorectal tissues showed the lowest. This indicates that there is very little neovascularization in normal colorectal tissue, but new blood vessels have gradually begun to appear in colorectal adenoma, which is a precancerous disease. In the evolution process of "normal intestinal epithelium → adenoma → cancer" of CRC, the microvessel density gradually increases. PLXDC1 and its homolog PLXDC2 are the only two proteins that have been shown to bind extracellular PEDF to the cell surface and to signal PEDF to the cell. They are a complete group of transmembrane proteins that are not only involved in cell-cell and cell-matrix interactions during capillary morphogenesis, but also in the process of capillary morphogenesis. They are also involved in the proliferation and maintenance of neovascular endothelial cells in the fibrovascular membrane[56]. Among them, PLXDC1, also known as tumor endothelial marker 7 (TEM 7), is a transmembrane cell indicator protein containing a plexiform protein domain[57]. In a gene expression dataset of endothelial cells isolated from solid tumors, PLXDC 1 was found to be overexpressed in endothelial cells from colon, breast, brain, and ovarian tumors[58]. Bagley *et al*[59] found that TEM-7 was highly enriched in the blood vessels of tumor tissues such as colon cancer, breast cancer, lung cancer, bladder cancer, ovarian cancer, and endometrial cancer, while it was rarely expressed in normal tissues and blood vessels. TEM-7 is a vascular protein related to angiogenesis. In addition, other studies have shown that the mean serum concentration of TEM7 in CRC patients is significantly higher than that in healthy controls, and TEM7 values gradually increase with the development of T, N and M stages. TEM7 serum concentration can be considered as a useful biomarker for detecting CRC patients, monitoring cancer progression and identifying patients with poor survival[60]. PLXDC2 is another receptor of PEDF, which is expressed in a variety of tumors such as hepatocellular carcinoma, gastric cancer, and CRC[61-63]. PLXDC2 receptor-mediated signaling is a direct effect of PEDF on cancer cells[56]. In this experiment, Spearman correlation analysis showed that

the expression intensity of PEDF was negatively correlated with the CD31-MVD value in both the colorectal adenoma group and the CRC group, and the CD31-MVD value increased with the decrease in PEDF expression. This suggests that PEDF plays a significant inhibitory role in the early events (adenoma stage) and later events (cancer stage) of CRC. When PEDF is highly expressed, the CD31-MVD value decreases, thereby inhibiting and reducing the formation of new blood vessels. The vascular inhibitory effect of PEDF is involved in the development of CRC. Combined with the above comprehensive analysis of the difference in PEDF expression in the three groups and its correlation with CD31-MVD, we can speculate that PEDF is a regulatory factor in the process of colorectal adenoma carcinogenesis. PEDF may be expected to be a new research target in the exploration of chemopreventive drugs to prevent and delay the carcinogenesis of colorectal adenoma and in the research of targeted drugs for the treatment of CRC.

The VEGF/VEGFR axis is indispensable for vessel angiogenesis and is a key driver of tumor vascularization. VEGF and VEGFR can regulate not only the generation of blood vessels that develop from precursor cells during the early embryonic period, but also the growth of blood vessels that are already present at later stages[64]. At the same time, the experiment also indicated a positive correlation between VEGF expression intensity and MVD in the CRC group, and the CD31-MVD value increased with the increase of VEGF expression. It is in line with the results of the majority of scholars' research[65]. The ratio of PEDF/VEGF finely regulates blood vessel formation, and the balance between the two plays a crucial role in angiogenesis[23,24]. PEDF can induce the expression of Fas ligand (FasL) in endothelial cells, and the apoptosis of endothelial cells can be induced by the binding of FasL to Fas receptor. However, high concentrations of anti-apoptotic proteins are present in normal vascular endothelial cells, which lead to the absence of Fas receptor expression. Thus, neovascular endothelial cells can be selectively inhibited by PEDF while still preserving the pre-existing vasculature, and PEDF has no effect on normal blood vessel formation[66]. In an *in vitro* model of angiogenesis, the inhibitory effect of PEDF on VEGF-induced

angiogenesis in the presence or absence of VEGF is mediated by enhancing the γ-secretase dependent C-terminal cleavage of VEGFR-1, thereby inhibiting VEGF-2-induced angiogenesis. In addition, PEDF regulates the phosphorylation of VEGFR-1, which itself regulates VEGFR-2 signaling. PEDF is a counteracting factor of VEGF and can inhibit VEGF-induced angiogenesis. The proposed underlying mechanisms of the biological effects of PEDF on endothelial cells involve the complex cross-talk between signaling events triggered by both proangiogenic and anti-angiogenic molecules[67]. In our experiment, PEDF and VEGF were correlated with CD31-MVD in the CRC group, indicating that PEDF and VEGF were both involved in the late event of the CRC stage. Furthermore, the correlation analysis revealed a negative relationship between PEDF expression intensity and VEGF expression intensity in the CRC group, and the expression intensity of VEGF decreased with the up regulation of PEDF expression. It is well known that as early as 2004, bevacizumab became the first VEGF-targeted therapy approved by the US Food and Drug Administration to treat metastatic CRC[49], and its effectiveness and safety have been confirmed. Currently, bevacizumab is still used as a first-line treatment drug for metastatic CRC[68]. Based on the result that the expression intensity of PEDF and VEGF is negatively correlated in the CRC group, we boldly speculate that in future targeted drug therapy, up-regulation of PEDF expression indirectly inhibits the expression of VEGF to inhibit tumor angiogenesis, which may provide a new idea for the treating of CRC.

In addition, in this experimental study, it was also found that there was no statistical difference in the correlation between the expression intensity of VEGF and CD31-MVD in the colorectal adenoma group, which was contrary to the results of Wang *et al*[69] in the early research. Wang *et al*[69] used the immunohistochemical method to to investigate the correlation between VEGF expression and MDV in 36 cases of adenoma specimens (including 12 cases of tubular adenoma, 12 cases of tubule-villous adenoma, and 12 cases of villous adenoma). The results showed that the MVD value in colorectal adenoma group was positively correlated with the expression intensity of VEGF (r = 0.640, $P < 0.01$). It is well known that the risk of canceration in colorectal adenomas

increases with histological progression[70], and in contrast, the canceration rate of tubular adenomas is relatively low compared with tubulovillous and villous adenomas. However, in the experimental adenoma group, 37 cases included tubular adenoma, accounting for 74%, while 11 cases were tubulovillous adenoma, accounting for 22%, and 2 cases were villous adenoma, accounting for only 4%. In the experimental adenoma group, tubular adenoma accounted for most. We speculate that the lack of correlation between VEGF expression and MVD in the adenoma group may be due to the imbalance in the proportion of adenomas with various histological features included in the adenoma group, but it may also be speculated that VEGF does not predominate in the angiogenesis of early adenomas. The reasons leading to the inconsistent results of previous studies can be further clarified and confirmed by enlarging the sample size and equalizing the proportion of adenomas with different histological characteristics in the adenoma group.

CONCLUSION

In summary, PEDF and VEGF are both involved in the occurrence and development of CRC during the evolution of the sequence of "normal intestinal epithelium → adenoma → carcinoma". PEDF is an inhibitory factor of CRC, and VEGF is a promoting factor of CRC. PEDF may be expected to be a new target for early prevention and late treatment of CRC. Up-regulation of PEDF expression and inhibition of VEGF expression may provide new ideas for targeted therapy for CRC.

ARTICLE HIGHLIGHTS

Research background

The morbidity and mortality of colorectal cancer (CRC) are among the highest in the world. When the balance between pigment epithelium-derived factor (PEDF), which inhibits angiogenesis, and vascular endothelial growth factor (VEGF), which stimulates angiogenesis, is broken, it can lead to uncontrolled angiogenesis and promote the

occurrence of tumors. Therefore, it is necessary to find more therapeutic targets for early intervention and late treatment of CRC.

Research motivation

The safety and efficacy of targeted drugs targeting VEGF in the treatment of CRC have been confirmed and promoted. PEDF is the anti-VEGF factor. At present, no toxicity caused by PEDF preparation itself has been observed in anti-tumor vascular animal models. It is worth exploring the possibility of PEDF as a new target for early prevention and late treatment of CRC.

Research objectives

Study of the expression and significance of PEDF, VEGF, and CD31-stained microvessel density values (CD31-MVD) in normal colorectal mucosa, adenoma, and CRC.

Research methods

We collected 50 cases of normal intestinal mucosa, 50 cases of colorectal adenoma and 50 cases of colon cancer as normal control group, adenoma group and CRC group, respectively. Immunohistochemical staining was used to detect the expression of PEDF and VEGF in the three groups, and the differences were analyzed. The relationship between the expression of PEDF and VEGF and the clinicopathological factors of CRC was studied. CD31-MVD was recorded in the three groups, and the correlation between PEDF, VEGF and CD31-MVD in colorectal adenoma group and CRC group was analyzed.

Research results

The positive expression rate and expression intensity of PEDF in normal control group, adenoma group and CRC group gradually decreased, while that of VEGF gradually increased. In the CRC group, the positive expression rate of PEDF decreased with the increase of differentiation degree, invasion depth, lymph node metastasis, distant

metastasis and TNM stage. The opposite was observed for VEGF high expression. In the colorectal adenoma group, the expression intensity of PEDF was negatively correlated with CD31-MVD, but there was no significant difference in VEGF expression. PEDF expression was negatively correlated with CD31-MVD and VEGF expression in CRC group. The expression of VEGF was positively correlated with CD31-MVD.

Research conclusions

It is possible that PEDF can be used as a new treatment and prevention target for CRC by upregulating the expression of PEDF while inhibiting the expression of VEGF.

Research perspectives

We will further expand our sample size to equalize the proportion of various types of adenomas in the colorectal adenoma group and the proportion of various pathological types of CRC in the CRC group to further confirm our conclusion.

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