International Journal of Biology and Life Sciences
ISSN: 2957-9511 | Vol. 1, No. 1, 2022

Preliminary study on angiogenesis and functional evaluation of benign adrenal pheochromocytoma vessels

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Abstract: Objective: To study the relationship between the pheochromacytomas' MVD and VEGF expression which are two indexes of angiogenesis, and analyze the difference of MVD and VEGF expression in different groups of pheochromocytomas with the purpose of correlating pheochromocytoma's function and angiogensis. Methods From June 2009 to December 2015, thirty-one patients histologically diagnosed as benign pheochromocytoma were divided into two groups according to the length of preparing time before operation(group 1 ≦2 weeks, group 2>2 weeks), and eight normal adrenal glands as the control group. The MVD and VEGF expression of all the pheochromocytomas and normal adrenal glands were evaluated, with the purpose of correlating the MVD and VEGF expression and analyzing the difference among of two groups of pheochromocytomas and normal adrenal glands. The tumors' diameter, intratumoral necrosis and the patients' urine VMA were also analyzed with MVD and VEGF expression. Results :1) The mean value of MVD in group 1 is 50.2±15.4 per HP, and less than the group 2 with the mean value 69.5±3.8 per HP($p<0.05$), but more than the normal adrenal glands with the mean value 35.1±3.4 per HP($p<0.05$). There is no MVD mean value difference between groups divided according to tumors' diameter, intratumoral necrosis and the patients' urine VMA. There is 30.8% high VEGF expression in group 1 less than group 2 with 77.8%($p<0.05$), and there no high VEGF expression rate difference between groups divided according to tumors' diameter, intratumoral necrosis and the patients' urine VMA; The MVD correlates with VEGF expression in pheochromocytomas ($r=0.545$, $p<0.05$). Conclusion :1) The function of pheochromocytoma maybe correlate with the angiogenesis. 2) The angiogenesis of pheochromocytoma was more than that of the normal adrenal gland. 3)There are no correlation with the angiogenesis and tumor's diameter, intratumoral necrosis and the patients' urine VMA in this study.

Keywords: Pheochromocytoma; Angiogenesis; MVD; VEGF; Function.

1. Introduction

Pheochromocytoma is a rare tumor, most of which are functional tumors. Because this kind of tumor secretes a large amount of catecholamine tissue, if it is not treated, the incidence rate and mortality of cardiovascular disease are very high. Therefore, adequate preoperative preparation is the key to the success of surgery for pheochromocytoma, which is not given routinely α- Before receptor blockers, the mortality of PHEO surgery was 24%~50%, and adequate drug preparation could lower the mortality of surgery to 3%. However, there is no uniform standard for the time of preoperative drug preparation and whether the volume expansion is sufficient. The time for capacity expansion preparation is generally 7-14 days, and it has also been reported that it takes 2-4 weeks or more. Therefore, in this study, we chose preoperative preparation time as an intervention factor to evaluate the difference of angiogenesis in different benign pheochromocytomas and the possible relationship between angiogenesis and tumor function.

Angiogenesis can be seen in a variety of physiological processes, such as wound healing, follicular and luteal formation. However, it is more involved in the occurrence and development of tumors in the pathological process. Angiogenesis is a dynamic process, so it is complicated to evaluate angiogenesis. Many studies evaluate angiogenesis by selecting a specific time point to study the result of angiogenesis, namely micro vessel density (MVD). Many studies have confirmed that MVD can be used as an independent factor to evaluate the prognosis of solid tumors. Vermeulen et al. proposed an international consensus, clarified the evaluation criteria of methodology and microvessel density, and hoped to increase the repeatability of research and the comparability of data between different research centers. At present, tumor angiogenesis can be evaluated by calculating tumor MVD, so as to evaluate tumor metastasis and prognosis. In addition, calculating tumor MVD also provides a basis for anti-tumor drug treatment.

Many clinical histopathological studies have shown that angiogenic factors are directly related to MVD. Among all angiogenic factors, vascular endothelial growth factor (VEGF) is one of the effective multifunctional cytokines. It is a glycoprotein with a molecular weight of 34~46KDa, a factor that can potentially stimulate the growth of endothelial cells, and can promote physiological or pathological angiogenesis, also known as vascular permeability factor (VPF). VEGF target cells are endothelial cells. On the one hand, VEGF makes microvascular permeability too high, leading to leakage of plasma protein and fibrinogen, which can stimulate angiogenesis and new matrix formation; On the other hand, VEGF stimulates microvascular endothelial cells to proliferate, migrate and change their gene patterns. VEGF and its receptors mainly regulate angiogenesis through paracrine or autocrine effects.

Pheochromocytoma, whether benign or malignant, is a tumor with rich blood supply. Many scholars evaluated the angiogenesis of pheochromocytoma by evaluating MVD and VEGF, hoping to find corresponding markers to evaluate the

benign and malignant tumors, and also provide basis for anti-tumor vascular therapy. In these studies, benign pheochromocytoma is the object of comparison, and its MVD value and VEGF expression are objective phenomena. Then, if benign pheochromocytoma is studied in subgroups, whether there will be differences in angiogenesis of these tumors, and whether these differences are related to their different functional states, is the question we want to discuss.

2. Materials and methods

2.1. Research object

The medical records of patients with adrenal pheochromocytoma who were hospitalized in the First Affiliated Hospital of Yangtze University from June 2009 to December 2020 and had complete clinical, pathological and follow-up data were collected. The patients with malignant pheochromocytoma, bilateral pheochromocytoma and static pheochromocytoma were excluded, and 31 patients were finally selected for inclusion. There were 9 males and 22 females, aged 37 to 73 years. Another 8 cases of benign renal diseases were taken for nephrectomy, and the normal adrenal tissue of the same side was taken as the control group.

2.1.1. Selection conditions

1.1.1.1 Pathologically confirmed pheochromocytoma, adrenal pheochromocytoma (pheochromocytoma of the adrenal gland scaled score, PASS) score<4, and the scoring standard is shown in Table 1-1; There was no tumor recurrence or metastasis during the follow-up period of 12 to 87 months; The patient was unilateral pheochromocytoma.

1.1.1.2 Criteria for patients with functional pheochromocytoma:

1)Preoperative patients had clinical manifestations of hypercatecholaminemia such as hypertension, tachycardia, hyperhidrosis and other symptoms, and other secondary diseases causing hypertension such as renal artery stenosis and sleep apnea syndrome were excluded.

2)Patients who were highly suspected of pheochromocytoma through clinical manifestations, biochemical examination and imaging examination before operation.

1.1.1.3 Preoperative preparation of patients

All patients received prazosin antihypertensive treatment before surgery (prazosin was mainly used for antihypertensive and volume expansion in our hospital before surgery). The initial dose of prazosin was 1mg tid, and gradually increased to 4mg tid to control hypertension. If the patient's blood pressure was still difficult to control, calcium channel blockers were added to control blood pressure; In addition, if the patient has tachycardia, add β Receptor blockers. The preoperative blood pressure control standard was 100-130/70-80 mmHg, and the heart rate was controlled at 60-80 beats/minute. The preoperative preparation time is calculated from the time when antihypertensive drugs are added to the time when blood pressure and heart rate reach the control standard. There was no hypertensive crisis or pheochromocytoma crisis during and after operation.

2.2. Source of specimen

Pathological specimens of 31 patients with adrenal pheochromocytoma who were surgically removed in our hospital from September 2009 to December 2020, being eligible for inclusion.

2.3. Main instruments and reagents

2.3.1. Main instruments

(1) Tissue slicer: leica biosystems, RM2245 Germany; (2) Optical microscope: OLYMPUS, BX53, Japan; (3) Micro vibrator: WZ-2A, Beijing Haidian Electronic Medical Instrument Factory; (4) Microwave oven: Japan Panasonic, NN-6599, Japan; (5) Pathological tissue bleacher: PHY - III, Changzhou Zhongwei Electronic Instrument Co., Ltd; (6) Blade: Crescent Manufacturing Company, D7223T; (7) Electric thermostatic blast drying oven: Shanghai Yuejin Medical Instruments Co., Ltd., GZX-GF101 - II; (8) Adhesive slide: Jiangsu Shitai Experimental Equipment Co., Ltd; (9) Cover glass: Jiangsu Shitai Experimental Equipment Co., Ltd; (10) Organization embedding box: Jiangsu Shitai Experimental Equipment Co., Ltd; (11) Neutral Gum: Shanghai Specimen Model Factory, China; (12) Moisturizing box: Beijing Zhongshan Biotechnology Co., Ltd; (13) Paraffin wax: Guangdong Maoming Dachuan Special Wax Factory Co., Ltd., GB/T254-1998.

2.3.2. Main reagent: CD31 mouse anti human monoclonal antibody

Thermo, JY-0029; VEGF rabbit anti human polyclonal antibody, Thermo, JY-0118.

2.4. Test method

2.4.1. Selection of wax block

The wax block shall be selected with well-preserved tumor tissue, and the tumor tissue shall contain capsule. The wax block with more tumor tissue shall be selected preferentially among several wax blocks.

First, call out the pathological sections of all patients, reevaluate the pathological sections of each patient, first evaluate the HE staining pathological sections, and then combine the results of several previous immunohistochemical antibodies against CgA, Syn, Melan-A, S-100, Ki-67, etc. to confirm that it is adrenal chromaffin tumor again. According to the PASS system, it was confirmed that the PASS score of pathological sections of the enrolled patients was less than 4 points. Observe all HE staining pathological sections of each patient, record the section number that meets the inclusion criteria, and select the wax block corresponding to the section number in the specimen room.

2.4.2. Test steps

(1) For the selected wax block, use a paraffin slicer to slice, with a slice thickness of 4 μ m. The surface of the tissue block shall be uniform, and the size and shape of the wax film cut shall be consistent with the shape of the tissue in the tissue block; (2) Place the paraffin slices in the bleacher and dryer to bake for 30 minutes to prevent peeling; (3) Put paraffin slices in two cylinders of xylene for dewaxing, and dewaxing for 5 minutes each; (4) After 30 seconds of anhydrous alcohol, 90% alcohol, 80% alcohol, 70% alcohol and 50% alcohol respectively; (5) After washing with double distilled water, it is washed three times with PBS buffer solution for 3 minutes each time, and then incubated in 3% H2O2 for 10 minutes to block the endogenous peroxidase activity; (6) The sections were put into EDTA PH8.0 buffer solution, and the antigen was repaired by microwave heating for 30 minutes; (7) After cooling at room temperature, wipe the liquid around the tissue on the slice and place it flat in a wet box, add 50-100 μ L of anti I to the tissue, and incubate it at room temperature for 2 hours; (8) Wash the tissue with PBS buffer for three times, 3 minutes each time, take out the slice, shake off and dry the

liquid around the tissue (do not dry the tissue slice), and place it flat in the wet box; (9) Add 50-100 μ L of secondary antibody GTVision I polymer, incubate at room temperature for 40 minutes; (10) Wash with PBS buffer for three times, 3 minutes each time, take out the slice, shake off and wipe the liquid around the tissue (do not dry the tissue slice), and place it flat in the wet box; (11) Add 50-100 μ L chromogenic agent DAB, incubate at room temperature for 5-10 minutes for color development; (12) Wash with distilled water to stop color development; (13) Rinse with running water for 3 minutes, then place it in hematoxylin dye solution for 3 minutes; (14) Rinse with running water for 3 minutes, and then put it into 1 ‰ hydrochloric alcohol for differentiation for 20 seconds; (15) Rinse with running water for 3 minutes, place it in 1% ammonia water and turn blue for 30 seconds; (16) After washing with running water for 3 minutes, place 70%, 95% alcohol, absolute alcohol, absolute alcohol and absolute alcohol for 30 seconds respectively; (17) Put them in two cylinders of xylene successively for 30 seconds; (18) Neutral gum sealing and baking. (19) Control design: positive control and blank control were set up, in which 0.01 mol/L PBS was used as blank control instead of the first antibody. Normal adrenal cortex was used as positive control for CD31, and placental tissue was used as positive control for VEGF.

2.5. Result judgment

2.5.1. MVD count in tumor

The counting was carried out by two pathologists who had studied the pathology of the adrenal gland. They did not know the clinical data and pathological results of the patients. The method of Weider et al. was used: positive structures such as vascular tissue, immunopositive cells and single endothelial cells were counted. If the vascular lumen area is greater than 8 red blood cells or the thickness of its muscle layer is large, it will not be counted. First, in the low power field of vision (× 10 Eyepiece, × 10 Objective lens) Select the area with the most blood vessels (hot spot area) in the tumor tissue, and then (× 10 Eyepiece, × Objective lens) Observe the number of blood vessels in 3 areas. Results Take the average value of each high-power visual field. Unit: Number of microvessels/high power visual field.

2.5.2. VEGF immunohistochemical staining

The evaluation was conducted by two pathologists who had studied the pathology of adrenal gland, and they did not know the clinical data and pathological results of the patient. The immune reaction of VEGF was judged as positive when the cytoplasm or membrane of tumor cells were brown yellow granules, and the degree was expressed as the sum of the grade and intensity of staining. The grade of staining is divided according to the percentage of positive cells: no cell staining=0, <10% cell staining=1, 10% - 50% cell staining=2,>50% cell staining=3. The intensity score of staining is: no staining=0 (basically no staining or the staining degree is similar to the background), slight staining=1 (not dark, but slightly higher than the background color), moderate staining=2 (obvious staining, and higher than the background color, brown yellow), and strong staining=3 (very obvious staining, dark brown yellow). The final result is the sum of the two scores, and the evaluation of VEGF immunoreactivity is within the range of 0-6 points. According to the score, 0~3 points were classified as low expression group and 4~6 points were classified as high expression group.

2.5.3. Record of hemorrhagic necrotic foci

The pathological sections of all patients were evaluated for the presence of hemorrhage and necrosis, and the corresponding records were made.

2.5.4. Training for counting and observers

Trained the judges of MVD count and VEGF expression results, informed them of the evaluation criteria, selected 10 pathological sections of patients to judge the results in advance, and required the two judges to have similar interpretation results of the same film to reduce human error.

2.6. Statistical judgment

The measurement data are first tested by normality test and homogeneity test of variance. The measurement data that conform to normal distribution and homogeneity of variance are expressed by mean ± standard deviation (± s), and the independent sample t-test is used for statistical analysis; The data that do not conform to the normal distribution and the homogeneity test of variance are tested by nonparametric test. Fisher exact test was used for counting data, and Spearman correlation analysis was used for correlation analysis. The difference was statistically significant with $P<0.05$.

3. Results

3.1. General situation of patients

This study included 31 patients, including 9 males and 22 females, aged between 37 and 73 years. Among 31 cases of unilateral adrenal pheochromocytoma, 17 were on the left and 14 on the right. The patients were all pheochromocytoma patients with clinical symptoms. The preoperative blood pressure fluctuated widely. Nine patients had coronary heart disease, arrhythmia, diabetes or abnormal glucose tolerance. The main qualitative and biochemical examination was urinary VMA, of which 17 patients had elevated urinary VMA, which was positive. All 31 patients received surgical treatment in our hospital, of which 24 patients underwent laparoscopic adrenalectomy and 7 patients underwent open surgery. One of the 7 patients switched to open surgery due to uncontrollable intraoperative bleeding. The patients were divided into two groups according to the preoperative preparation time, namely ≤ 2 weeks group (group 1) and>2 weeks group (group 2).

3.2. Pathology of patients

Postoperative HE staining and immunohistochemical results confirmed that it was pheochromocytoma (see Figure 1), and the immunohistochemical results of Ki67 in all patients were within 10% (see Figure 2). Through PASS score, no patient with pheochromocytoma score greater than or equal to 4 was found. The tumor diameter was 1 cm~20 cm, including 11 cases with tumors over 5 cm and 20 cases with tumors less than 5 cm; There were 7 cases of tumor with hemorrhage and necrosis, and 5 of the 7 cases were larger than 5 cm in diameter.

3.3. Angiogenesis (MVD, VEGF expression)

CD31 is expressed in most capillaries, and the area with the highest microvessel density count expressed and labeled by CD31 is about 1mm below the edge of the tumor capsule tissue. There are differences in expression between normal adrenal tissues and different benign pheochromocytomas (see Figures 3, 5, 6, and 7). The mean MVD in the group with preoperative preparation time ≤ 2 weeks was 50.2 ± 15.4 pieces/HP, which was significantly lower than that in the group with preoperative preparation time>2 weeks (69.5 ± 3.8

pieces/HP) and higher than that in the group with normal adrenal gland (35.1 ± 3.4 pieces/HP) (see Table 1for details). In addition, there are differences in the shape and course of blood vessels in different tumor tissues. The course and shape of some blood vessels can be observed in a small number of tissue sections (see Figure 8).

Table 1. MVD count of pheochromocytoma and normal adrenal tissues in different preoperative preparation time groups

	Number of cases	MVD (strip/HP)
Preparation time ≤ 2 weeks	13	50.2±15.4
Preparation time > 2 weeks	18	69.5±3.8
Normal adrenal group	8	35.1±3.4

Note: The MVD value of the group with preoperative preparation time ≤ 2 weeks was compared with that of the group with preoperative preparation time>2 weeks (P<0.05), and the MVD value of the group with pheochromocytoma and normal adrenal gland was compared with that of the group with preoperative preparation time ≤ 2 weeks (P<0.05).

There are differences in the strength and grade of VEGF expression in different tumor tissues (see Figure 9, Figure 10, and Figure 11). The mean MVD in the low VEGF expression group was 47.0 ± 11.1 bars/HP, which was lower than 71.8 ± 15.4 bars/HP in the high VEGF expression group, with a significant difference (see Table 2). In addition, Spearman rank correlation analysis showed that VEGF and MVD were correlated, with correlation coefficients of r=0.545 and P=0.02.

Table 2. Comparison of MVD values between low VEGF expression group and high VEGF expression group

	Number of cases	MVD(strip/HP)
VEGF low express group	13	47.0±11.1
VEGFhigh express group	18	71.8±15.4

Note: The mean value of the two groups was P<0.05.

In benign pheochromocytoma, the expression of VEGF was different in different preoperative preparation time groups. In ≤ 2 weeks group, the expression of VEGF was low in 9 cases, accounting for 69.2%; in>2 weeks group, the expression of VEGF was low in 4 cases, accounting for 22.2%; while in normal adrenal group, the expression of VEGF was low (see Figure 3. Among them, Fisher exact test was used, and the VEGF expression in the two groups with different preoperative preparation time was significant (P=0.013).

Table 3. Expression of VEGF in different preoperative preparation time groups and normal adrenal tissues

	Number of cases	VEGF Low expression	VEGF High expression
Preparation time ≤ 2 weeks	13	9(69.2%)	4(30.8%)
Preparation time>2 weeks	18	4(22.2%)	14(77.8%)
Normal adrenal group	8	8(100%)	0(0%)

Note: The expression of VEGF in different preoperative preparation time groups was different (P<0.05).

3.4. Differences in angiogenesis of pheochromocytoma with different pathological and biochemical characteristics

In the pathological analysis of pheochromocytoma, there were ischemia and necrosis in some tumors, as well as differences in tumor diameter. In order to study the difference of tumor angiogenesis with different pathological characteristics, the tumor was divided into two groups according to whether there was necrosis and the longest diameter of the tumor was 5cm.

The patients in this group were qualitatively diagnosed as urinary VMA before surgery, so they were divided into two groups according to the positive and negative urinary VMA, so as to compare the angiogenesis of tumors with different biochemical indicators.

Among them, the proportion of VEGF overexpression in tumor necrosis group was higher (83.3%), while that in non-necrosis group was 50%, but there was no significant difference between Fisher's exact test and non-necrosis group (P=0.191). The MVD value of tumor in the same two groups was 69.3 ± 14.2/HP in the necrotic group, which was higher than 62.0 ± 19.7/HP in the non-necrotic group, but the difference was not significant (P=0.740). (See Table 4 for details)

Table 4. Relationship between tumor necrosis and angiogenesis

	Number of example	VEGF Low expression	VEGFHigh expression	MVD(strip/HP)
Tumor necrosis group	7	1(16.7%)	6(83.3%)	69.3±14.2
Tumor without necrosis group	24	12(50%)	12(50%)	62.0±19.7

Note: The expression of VEGF in the two groups was P>0.05, and the mean MVD was P>0.05.

The proportion of VEGF overexpression in pheochromocytoma with a diameter of ≥ 5cm was higher, accounting for 71.8%, while that in pheochromocytoma with a diameter of<5cm was 45.0%, but there was no significant difference between the two groups (P=0.066). The mean MVD of pheochromocytoma with diameter ≥ 5cm was 66.8 ± 20.7 pieces/HP, which was higher than 58.4 ± 16.8 pieces/HP of pheochromocytoma with diameter<5cm, but the difference was not significant (P=0.23). (See Table 1-6 for details)

Table 5. Expression differences of MVD and VEGF in pheochromocytomas of different diameters

	Number of example	VEGF Low expression	VEGFHigh expression	MVD(strip/HP)
Group with diameter<5cm	20	11(55.0%)	9(45.0%)	58.4±16.8
Group with diameter ≥ 5cm	11	2 (18.2%)	9(71.8%)	66.8±20.7

Note: The expression of VEGF in the two groups was P>0.05, and the mean MVD was P>0.05.

The proportion of VEGF overexpression in urine VMA positive group was 64.7%, while that in urine VMA negative group was 50.0%. Although the positive rate of VEGF overexpression in urine VMA positive group was higher than

that in urine VMA negative group, the difference was not significant (P=0.481). The mean MVD value of urine VMA positive group was 63.5 ± 19.0 pieces/HP, which was higher than 58.9 ± 18.0 pieces/HP of urine VMA negative group, but compared with the two groups, P=0.484. (See Table 6 for details)

Table 6. Relationship between urinary VMA and MVD, VEGF expression in tumor tissue

	Number of examples	VEGF Low express	VEGFHigh express	MVD (strip/HP)
Urine VMA positive group	17	6(35.3%)	11(64.7%)	63.5±19.0
Urine VMA negative group	14	7(50.0%)	7(50.0%)	58.9±18.0

Note: Comparison of VEGF expression between the two groups P>0.05, MVD mean comparison, P>0.05.

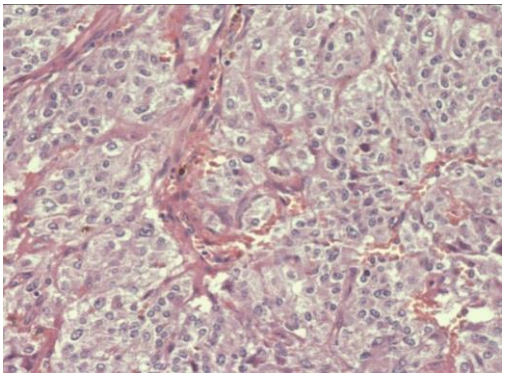

Fig. 1 HE staining of pheochromocytoma (× 200)

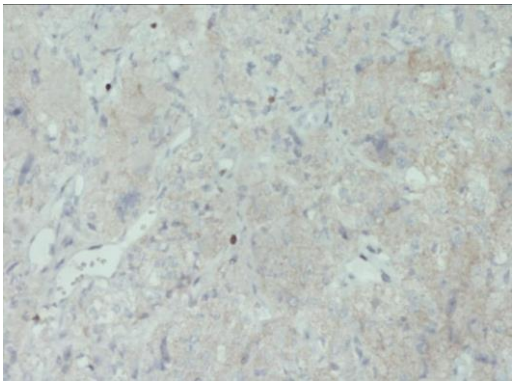

Fig. 2 Ki-67 positive rate<5% (× 200)

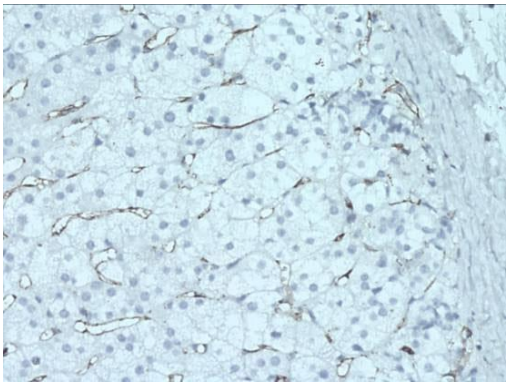

Fig. 3 Immunohistochemical staining of CD31 in adrenal tissue, low MVD count (× 200)

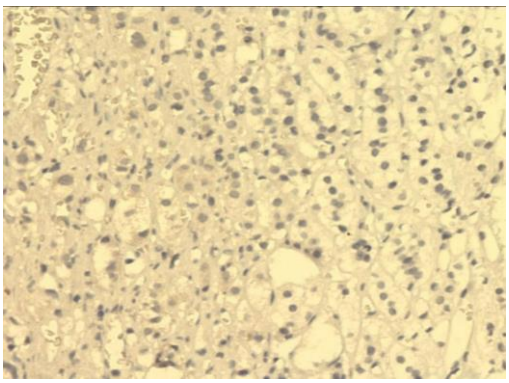

Fig. 4 Low expression of VEGF in adrenal tissue (× 200)

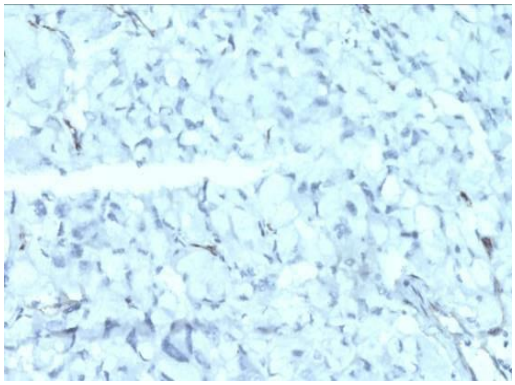

Fig.5 CD31 immunohistochemical staining in pheochromocytoma, MVD count is less (× 200)

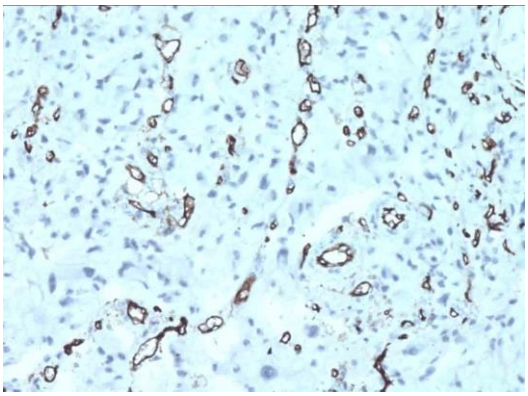

Fig.6 CD31 immunohistochemical staining in pheochromocytoma, with medium MVD count (× 200)

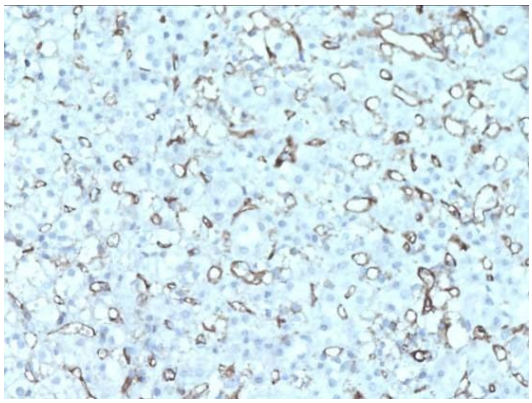

Fig. 7 CD31 immunohistochemical staining in pheochromocytoma shows high MVD count (× 200)

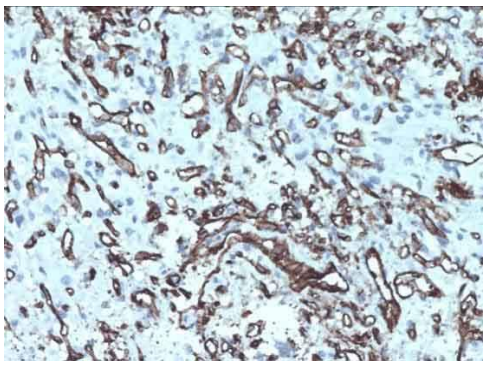

Fig. 8 Immunohistochemical staining of CD31 in pheochromocytoma tissue, abnormal vascular routing and morphology (× 200)

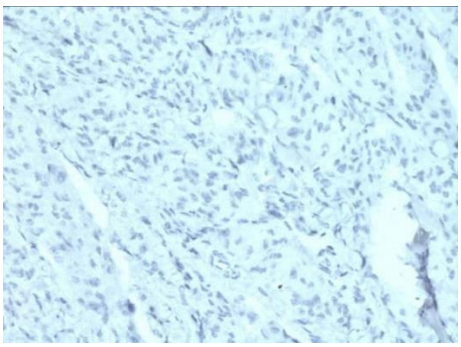

Fig.9 VEGF expression is negative in pheochromocytoma tissue (× 200)

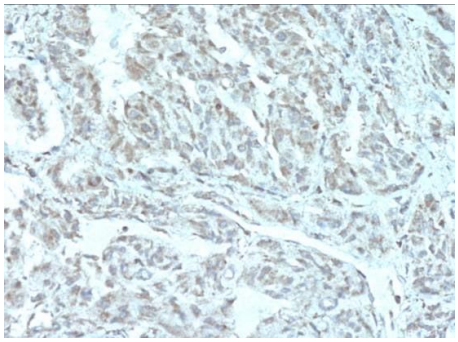

Fig. 10 VEGF expression in pheochromocytoma tissue is weakly positive (× 200)

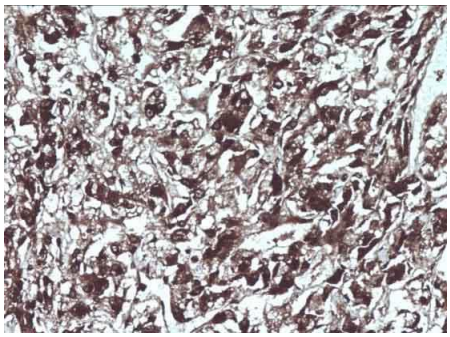

Fig. 11 VEGF expression in pheochromocytoma tissue is strongly positive (× 200)

4. Discussion

4.1. Clinical functional grouping of pheochromocytoma and inclusion criteria of benign pheochromocytoma

Pheochromocytoma is a tumor produced by chromaffin tissue from neural crest. Because it can produce and secrete a large amount of catecholamine tissue, causing changes in the circulatory system and metabolism, and even causing severe cardiovascular and cerebrovascular complications and serious metabolic disorder in patients, even if the tumor is benign, it is also a potentially dangerous tumor. The clinical manifestations of patients with pheochromocytoma are very different. Classification based on clinical manifestations is lack of quantitative criteria, which cannot fully evaluate the function of pheochromocytoma. At present, there is no widely accepted evaluation method. Pan Dongliang et al, the function of pheochromocytoma was divided into four grades according to the urine catecholamine level and fingertip microcirculation image, so as to guide the preoperative preparation. In this study, patients were divided into pheochromocytoma groups with different functions according to their preoperative preparation time. Due to the limitation of the experimental design, the number of samples included in the study was small, and the number of samples larger than 4 weeks was small (3 cases), so they were not grouped separately. Therefore, the functional pheochromocytoma group was divided into two groups: the group with more than 2 weeks and the group with less than 2 weeks. At present, there is no intuitionistic quantitative standard to judge whether the volume expansion is sufficient before operation. It is generally estimated by clinical manifestations such as normal blood pressure, stable heart rate, weight gain, nasal congestion and warm hands. The specific indicators that can be used for reference to judge adequate preoperative preparation include: no typical symptom attack within one week, slight nasal congestion, no sense of cold or warmth at the extremities, and ruddy nail bed, indicating good microcirculation perfusion; The blood pressure is stable at 120/80mmHg, and the heart rate is less than 80 beats/minute; No paroxysmal hypertension, palpitation, hyperhidrosis and other clinical manifestations; Body weight tends to increase, hematocrit<45%, or decrease by more than 5% . Because some patients are complicated with cardiovascular and cerebrovascular diseases, the basic conditions of each patient are different, and the blood pressure fluctuates, we set the preoperative blood pressure control standard as 100~130/70~80mmHg within 2~3 days, and the intraoperative blood pressure and heart rate are within the control of the anesthesiologist, and there is no pheochromocytoma crisis during and after the operation. This grouping method is judged by the treatment results, which can more fully evaluate the tumor function, but it is a retrospective judgment, and the evaluation method of the above research function is preoperative pre-judgment.

The differential diagnosis between benign and malignant pheochromocytoma is currently lack of specific diagnostic basis in clinical manifestations and histopathology. It is difficult to distinguish between benign and malignant pheochromocytoma. At present, the more accurate diagnostic standard for malignant pheochromocytoma is to show tumor metastasis and recurrence at the location without chromaffin tissue. The PASS scoring standard was proposed in 2002 and applied to the differential diagnosis of benign and malignant pheochromocytomas. The scoring system scores the pathological characteristics of multiple tumor cells. It is believed that tumors with more than 4 points tend to be more malignant. The sensitivity of the scoring system can reach 100%, and the specificity is 75%. However, the scoring system has some shortcomings. It is difficult to judge a large number of pheochromocytomas with PASS of 4-5 points as

benign or malignant, The accumulation and constant correction of clinical cases with larger samples are also needed. In view of this, we used this scoring standard for reference when we enrolled the study cases, and the PASS score of the included cases was less than 4 points. In addition, Ki67 is routinely detected in the pathology of the patients we enrolled. This indicator and mitotic image are used to grade neuroendocrine tumors, so as to judge the malignant degree of tumors. Among them, neuroendocrine tumors are considered for tumors with Ki67 ≤ 20%, while neuroendocrine tumors are considered for tumors with Ki67>20%. This indicator can be used to judge the reproducibility of neuroendocrine tumors, so it can be used to evaluate the prognosis of patients. In this study, Ki67 index of all patients was lower than 20%. We combined the above two indicators and the follow-up results to determine whether the enrolled cases were benign. Due to the needs of the study design, pheochromocytoma cases with high potential malignancy, such as ectopic pheochromocytoma, bilateral pheochromocytoma and familial pheochromocytoma cases, were not included in the study.

4.2. Evaluation of the angiogenesis

Angiogenesis refers to the process of forming new capillaries from preexisting vascular networks, which is involved in physiological processes such as embryonic development and wound healing, but also in tumorigenesis, where solid tumor growth, infiltration, and formation of metastasis depend on neovascularization.Tumor growth, metastasis depend on angiogenesis, and when it grows to 2 to 3 m m 3, its further growth is dependent on neovascularization,while VEGF is the most potent and most specific angiogenic factor known, acting mainly through the KDR / flk-1 receptor.At present, the most commonly used in the evaluation of angiogenesis are the tumor microvessel density (MVD) count and the expression degree of angiogenic factors such as VEGF, among which MVD is considered as the "gold standard" for the evaluation of tumor neovascularization.

Many scholars have chosen different antibodies to evaluate the MVD within different tumors, and the commonly used antibodies are CD34, CD31, and CD105. As an endothelial cell marker, the CD31 antibody has obvious and definite results of , and can easily be clearly observed and counted, and both large and small vessels can show an equal degree of positive .Therefore, in this study, we chose the CD31 antibody to evaluate the MVD of the tumors. Count MVD first needs to select tumor vascular hotspots, including manual counting method, Chalkley counting method and computer image analysis system counting method.The computational methods are constantly correcting and improving . We used the classical method of Weider and the method of manual counting to select hot spots for MVD evaluation. Correct identification of tumor vascular hotspots and observer experience are the two most important factors. Therefore, we took the pre-experiment and trained the observers to reduce the influence of subjective factors.

The most widely studied family in the VEGF family is VEGF-A, and usually VEGF immediately refers to VEGF-A. In benign and malignant pheochromocytoma tissues, VEGF-A expression is much higher than VEGF-C and VEGF-D expression, and VEGF-C and VEGF-D appear to be rarely involved in generation in pheochromocytoma blood vessels.At present, the antibodies to evaluate VEGF expression are monoclonal antibodies and polyclonal antibodies.Different studies use antibodies differently, so the VEGF expression has different . In this study, we selected polyclonal antibodies, and the experimental results confirmed that the VEGF expression was higher than that reported in the literature with monoclonal antibodies. Meanwhile, the correlation between MVD and VEGF expression was also confirmed in our study of benign pheochromocytoma.

4.3. Study of angiogenesis in pheochromocytoma

Currently, many scholars have conducted a large number of studies on tumor markers, many of which are related to the angiogenesis of pheochromocytoma. Counting of pheochromocytoma MVD and the evaluation of VEGF expression are widely used in the studies of malignant pheochromocytoma. Feng et al through the analysis of pathological and clinical data of 136 patients with pheochromocytoma, found that the expression of three angiogenesis-related factors, VEGF, COX-2 and MVD, was significantly higher in malignant pheochromocytoma patients compared with benign tumors.Favier et al found hereditary pheochromocytoma,such as angiogenic and major proangiogenic factors in tumors associated with SDH and VHL gene-related tumors,including VEGF and its receptors as well as HIF-2 α, provascular protein factor 2, endothelin receptor ETA and ETB were all significantly higher than RET, NF1 and TMEM127-associated tumors as well as sporadic pheochromocytoma.Therefore, anti-VEGF treatment is recommended for this type of malignant pheochromocytoma.However, some other scholars have found that benign and malignant pheochromocytoma MVD is not significantly different .It may be related to the sample size of the experimental cases, the MVD counting method used and the antibodies used.

Both benign and malignant pheochromocytomas are vascular-rich tumors.In the above study, although the investigators mainly focused on the comparison and identification of benign and malignant pheochromocytoma, but through these data, we found differences in MVD values and the degree of VEGF expression in different benign pheochromocytoma specimens.So as benign tumors, these angiogenesis differences with those factors is worth attention and study.At present, the research focus of pheochromocytoma angiogenesis is mainly focused on the diagnosis of malignancy, and anti-tumor vascular treatment, and on exploring the methodology adopted in these studies.In fact, angiogenesis is involved in many physiological processes and other pathological processes, including the formation of benign tumors.In the angiogenesis studies of adrenal disease,we find that both MVD and VEGF expression of benign adrenal adenomas and non-adenomas including pheochromocytoma and hyperplastic nodules are different.The growth of tumors requires the blood to provide nutrition and oxygen, but the tumor secretion function is also inseparable from the blood supply.Bernini et al studied the angiogenesis of normal adrenal cortex and adrenal cortical diseases, and found that the MVD of cortical cancer was significantly lower than that of adrenal cortex, aldosterone adenoma, cortisol adenoma and nonfunctional adenoma, and MVD decreased in turn in the latter four;In the case of VEGF expression, adrenocortical carcinoma is higher than aldosterone adenoma, cortisol adenoma, normal adrenal cortex and nonfunctional adenoma, and the latter four VEGF

expression is also reduced in turn.In addition, MVD values were positively correlated with aldosterone levels and negatively correlated with renin levels.Therefore, the authors believe that: adrenocortical carcinoma MVD and VEGF expression are inconsistent, and the functional status of nonfunctional adenoma is associated with its low MVD and low VEGF expression, while the secretion function of aldosterone adenoma is related to angiogenesis, indicating that angiogenesis may affect the hormone secretion function of endocrine tumors.Whether the different MVD and VEGF expression of benign pheochromocytoma is associated with its secretory function is the direction of our study. In the enrolled cases, we used α receptor and β receptor for adequate preoperative preparation, grouped pheochromocytomas according to the length of preparation time, and in the study we found higher MVD and VEGF expression in the preoperative preparation time group> 2 weeks than in the 2-week group.Based on this, we speculate that the benign pheochromocytoma secretory function is associated with its angiogenesis.

Tumor microvascular morphology often occurs some abnormal changes, the main manifestations are: blood vessel diameter thickness, irregular vascular morphology with distortion and structural disorder, visible vascular network, vascular pool and vascular lake, vascular wall is incomplete, base and basement membrane lack, endothelial cell gap expansion, tumor vascular lack of innervation.Favier et al reported different vascular structures of benign and malignant pheochromocytoma: benign tumor blood vessels are relatively regular small blood vessels, while malignant tumors have irregular blood vessels arrangement, and some larger blood vessels travel between different tumor nodules.Some scholars have analyzed the vascular structure of benign and malignant pheochromocytoma, and found that the benign and malignant tumors cannot be judged only by the vascular structure type of pheochromocytoma.Bialas et al , in his study, also found some tumor vascular structural abnormalities in a fraction of pheochromocytomas,whether benign and malignant.In our study, we also observed different benign pheochromocytomas, and their vascular morphology and walking were also different, and some pheochromocytomas vascular structure and walking are highly irregular (see Figure 8).So what is relevant to these vascular structure abnormalities in pheochromocytoma is a question to be further explored.

In the results of this study, we found that MVD and VEGF expression of pheochromocytoma with ischemia necrosis compared with the group without ischemia necrosis, although not significant difference, but higher than the group without ischemia necrosis.Tumor necrosis is associated with tumor hypoxia, angiogenesis and inflammatory response of . Hypoxia in the tumor microenvironment can induce increased VEGF expression and enhanced VEGF activity. The specific mechanism is related that hypoxia can activate VEGF gene transcription and increase VEGF mRNA stability, while hypoxia also enhances VEGF receptor transcription.Among them, the hypoxia-inducible factor-1 (HF-1) may play an important role in the transcription process of VEGF. Data from this group showed no difference in angiogenesis in the two groups of pheochromocytoma, which may be related to the small sample size.

There is much discussion about the size of adrenal pheochromocytoma and its function and benign and malignant.Some researchers have compared the clinical data of benign and malignant pheochromocytoma,they found the malignant pheochromocytoma was larger in diameter and in weight than the benign pheochromocytoma,Malignant pheochromocytoma plasma deethoxyladrenaline, perioperative 24-h urinary demethoxyladrenaline or non-dephenylepinephrine levels were all higher than benign pheochromocytoma,and the MVD value and VEGF expression of malignant pheochromocytoma were higher than that of benign pheochromocytoma .The above studies suggest that the larger tumor diameter, the greater malignancy, while increased angiogenesis.And whether there is a link between tumor diameter and angiogenesis then needs further confirmation.From the results of this study,large diameter tumors, compared with smaller diameter tumors showed slightly higher MVD and VEGF expression, but no significant difference.At the same time, in the study, we found that the proportion of large diameter tumors undergoing necrosis is very high, and intra-tumor necrosis may stimulate the expression of VEGF, thus increasing angiogenesis. Even if some tumors have no obvious necrosis, it may be because the tumor is too large, and local tissue ischemia and hypoxia, which can stimulate angiogenic.

Urinary VMA measurement is considered to enable a more accurate evaluation of pheochromocytoma functional, but the presence of a false-negative urine VMA possibility. In 31 patients in this group, only 17 patients were positive for urinary VMA. In our study, the urinary VMA positive patients found no significant difference between negative patients and angiogenesis, which may have some relationship with false negative urinary VAM.

In conclusion, in the present study, we found a correlation between MVD and VEGF expression in the benign pheochromocytoma group;The expression of benign pheochromocytoma MVD and VEGF varies in different preoperative preparation time groups, and the status of pheochromocytoma angiogenesis and its secretory function may be correlated.There are differences in the vascular structure between different pheochromocytomas, and whether the MVD and VEGF expression are related to the necrosis and diameter of the tumor needs further further investigation and exploration.

4.4. Conclusion

In benign pheochromocytoma, MVD is associated with VEGF generation, and angiogenesis was higher in benign pheochromocytoma than in normal adrenal tissue.②MVD and VEGF expression of angiogenesis in pheochromocytoma are different in different preoperative preparation time groups, and benign pheochromocytoma function may be related to angiogenesis.③In this study, benign pheochromocytoma with tumor necrosis was too high, but it was not significantly different from tumors without necrosis.MVD and VEGF expression in diameter> 5cm benign tumors tended to increase compared with diameter <5cm tumors, but the difference was not significant.Whether the above factors are associated with angiogenesis requires further study.④There exists atypia between vascular progression and morphology of some benign pheochromocytoma.

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