

# Correlation between microvessel density (MVD) and multi-spiral CT (MSCT) perfusion parameters of esophageal cancer lesions and the diagnostic value of combined CtBP2 and P16<sup>INK4A</sup>

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**Background:** This article aims to analyze the correlation between microvessel density (MVD) and multispiral CT(MSCT) perfusion parameters of esophageal cancer lesions, and the diagnostic value of combining C-terminal binding protein 2 (CtBP2) and P16 inhibitor of cyclin-dependent kinase 4a (P16<sup>INK4A</sup>).

**Methods:** A total of 42 cases of normal esophageal mucosa tissues >5 cm from the cancer tissue were selected as the control group. The expression levels of CtBP2 and P16<sup>INK4A</sup> and the values of MSCT perfusion parameters and MVD were compared in the control group and esophageal cancer group. SP immunohistochemical staining was used to detect protein expression levels of CtBP2 and P16<sup>INK4A</sup>. The Pearson method was used to analyze the differences and pertinence of MSCT perfusion parameters and MVD in the control group and esophageal cancer group. The receiver operating characteristic (ROC) curve was used to calculate the diagnostic value of CtBP2 and P16<sup>INK4A</sup> combined with MVD and MSCT perfusion parameters in esophageal cancer.

**Results:** The positive expression rate of P16<sup>INK4A</sup> in the esophageal cancer group was significantly lower than that in the control group. The positive expression rates of CtBP2, blood volume (BV), mean transit time (MTT), surface permeability (permeability surface, PS), and MVD values were significantly higher than those of the control group (P<0.05). There was no significant difference in blood flow (BF) value between the 2 groups (P>0.05). The BF value of the tumor invading the fibrous membrane was significantly higher than that of the non-invading fibrous membrane (P<0.05), and the PS and MVD values of the patients with lymph node metastasis were higher than those without lymph node metastasis (P<0.05). The MSCT perfusion parameters BF and BV were significantly positively correlated with MVD (P<0.05), while MTT, PS, and MVD were not significantly correlated (P>0.05). ROC results showed that the areas under curve (AUC) of CtBP2, P16<sup>INK4A</sup>, and MSCT were 0.625, 0.747, and 0.812, respectively. However, the area under the combined detection curve was larger, at 0.869.

**Conclusions:** MSCT perfusion imaging of esophageal cancer lesions can indirectly reflect the angiogenesis of esophageal cancer, and the combination of CtBP2 and P16<sup>INK4A</sup> can effectively improve the diagnostic efficiency of the disease.

**Keywords:** Esophageal cancer; multi-spiral CT (MSCT); microvessel density (MVD); P16 inhibitor of cyclindependent kinase 4a (P16<sup>INK4A</sup>); C-terminal binding protein 2 (CtBP2)

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### Introduction

Esophageal cancer is a malignant tumor that originates from the epithelium of the esophageal mucosa, accounting for 2% of all malignant tumors (1). The basis of tumor development and metastasis is closely related to tumor angiogenesis, the cell cycle negative regulator P16<sup>INK4A</sup>, and the transcription inhibitor C-terminal binding protein 2 (CtBP2) (2). Tumor microvessel density (MVD) is the "gold standard" that reflects tumor neovascularization, but the determination of MVD depends on living tissue. With the popularity of multi-spiral CT(MSCT), MSCT perfusion imaging has been widely used in a variety of organs and tumors, which can quantitatively reflect the characteristics of lesions or tumor microcirculation (3). High MVD is a prognostic factor among esophageal cancer that indicated worse prognosis in these patients (4). The abnormal expression and modification of CtBP2 and P16 inhibitor of cyclindependent kinase 4a (P16<sup>INK4A</sup>) play an important role in the occurrence of esophageal cancer. CtBP2, as a transcriptional corepressor of epithelial-specific genes, contributes to malignant development of human esophageal squamous cell carcinoma by regulation of p16<sup>INK4A</sup> (5). Cyclin H (CCNH)/ cyclin-dependent kinase 7 (CDK7)-CtBP2 axis may augment ESCC cell migration (6). Aberrant methylation of p16INK4a and deletion of p15INK4b are frequent events in human esophageal cancer in Linxian, China (7). Previous studies have found that the expression of CtBP2 and P16<sup>INK4A</sup> has certain differences in esophageal benign and malignant diseases, which is of great significance for disease diagnosis (8). However, there are few reports regarding MVD and MSCT perfusion parameters combined with CtBP2 and P16<sup>INK4A</sup> in the diagnosis of esophageal cancer. Therefore, this study aims to provide a reliable basis for clinical diagnosis and treatment by analyzing the diagnostic value between MVD and MSCT perfusion parameters combined with CtBP2 and P16<sup>INK4A</sup> in esophageal cancer. We present the following article in accordance with the STARD reporting checklist (available at http://dx.doi.org/10.21037/jgo-21-247).

### Methods

### General information

The clinical data of 103 patients with esophageal cancer admitted to our hospital from January 2019 to January 2021 were collected. All patients agreed to participate in this

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study and signed an informed consent form. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Henan Provincial Chest Hospital (No. 20181224). The inclusion criteria were as follows: (I) all patients met the relevant diagnostic criteria for esophageal cancer (9); (II) no history of allergies; (III) no history of esophageal cancer related radiotherapy and chemotherapy; (IV) no contraindications for CT examination. The exclusion criteria were as follows: (I) combined with any other tumor diagnosis history; (II) combined with immune function and blood dysfunction; (III) severe renal insufficiency; (IV) missing clinical data.

Finally, a total of 91 patients were enrolled, including 55 males and 36 females. The ages ranged from 44 to 65 years old, with an average of 57.69±8.14 years old. In terms of pathological types, there were 17 cases of adenocarcinoma and 74 cases of squamous cell carcinoma. There were 62 cases of high-medium differentiation and 29 cases were poorly differentiated. In terms of clinical staging, there were 15 cases in stage I, 36 cases in stage II, and 40 cases in stage III, and 71 cases had lymph node metastasis. A total of 42 cases of normal esophageal mucosa tissues >5 cm from the cancer tissue were selected as the control group. It was confirmed pathologically that there was no tumor cell infiltration, inflammation, cell proliferation, and other pathological changes.

### MSCT examination

The American GE 64-row CT machine was employed to detect relevant indicators. During the scan, the patient needed to breathe calmly and never swallow. The scan range was from the entrance of the patient's neck thorax to the plane of the stomach fundus. The scanning parameters were tube voltage of 120 kV, tube current of 120 mA, scanning layer thickness of 5 mm, thread pitch of 1.0, and reconstruction layer thickness of 2 mm. The patient lay flat on the scanning bed in the supine position. A conventional plain scan + 50 mL iohexol enhanced scan was usually used, and the injection rate of cubital vein injection was 4 mL/s. After the scan was completed, the CT post-processing workstation was used to reconstruct the coronal and sagittal images of the patient's axial scan. Corresponding blood flow (BF) and blood volume (BV), mean transit time (MTT), and surface permeability (permeability surface, PS) were measured. Three areas were selected, then the perfusion related parameters and their average value were obtained.

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Crown	CtBP2, n (%)		P16 <sup>INK4A</sup> , n (%)		
aloup	Positive	Negative	Positive	Negative	
Esophageal cancer group (n=91)	51 (56.04)	40 (43.96)	23 (25.27)	68 (74.73)	
Control group (n=42)	8 (19.05)	34 (80.95)	30 (71.43)	12 (28.57)	
$\chi^2$	15.936		25.538		
Р	<0.001		<0.001		

Table 1 Comparison of CtBP2 and P16<sup>INK4A</sup> expression in the control group and esophageal cancer group

CtBP2, C-terminal binding protein 2; P16<sup>INK4A</sup>, P16 inhibitor of cyclin-dependent kinase 4a; P value, probability;  $\chi^2$ -test, Chi-square test.

# CtBP2 and P16<sup>INK4A</sup> detection

The main reagents included mouse anti-human CtBP2 monoclonal antibody (U.S. Neomarkers company) and rabbit anti-human P16<sup>INK4A</sup> polyclonal antibody (Wuhan Boster Biotechnology Co., Ltd., China). The streptavidin-peroxidase ligation (SP) kit, trypsin, and the diaminobenzidine substrate color kit were purchased from Fuzhou Maixin Company.

The esophageal cancer tissue was fixed with 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned. Paraffin sections were separated in xylene and rehydrated in gradient ethanol. According to the instructions, SP immunohistochemical staining was used to detect protein expression levels of CtBP2 and P16<sup>INK4A</sup>. The negative control of the primary antibody was replaced by phosphate buffered saline (PBS).

The results were judged based on the percentage of positive cells and the intensity of staining (10). (I) The positive standard was the bleeding of brown or brown particles in the nucleus or cytoplasm. Zero points were for colorless, 1 point for grayish yellow, 2 points for golden yellow, and 3 points for brown. The total score was the product of the above 2 scores. A positive meant that the total score was >4 points. (II) The positive cell rate calculation involved the random selection of 5 high-power fields from each slice and calculating the number of positive cell number ×100%. Zero points indicated no positive cells, 1 point indicated positive cells <10%, 2 points was 10–50%, 3 points was 50–75%, and 4 points was >75%. The total score was the product of the above 2 scores. A positive meant that the total score was >4 points was >75%.

### MVD count

Low-power microscopy (×100) was used to observe the SP-stained sections. The most stained vascular endothelial

cells were selected in the field of view. High-power microscopy ( $\times$ 400) was used to count the number of microvessels in the 3 fields, and then the average value was taken as the MVD value.

### **Observation indicators**

(I) The expression levels of CtBP2 and P16<sup>INK4A</sup>, MSCT perfusion parameters, and MVD were compared in the control group and the esophageal cancer group. (II) The different pathological characteristics and the correlation between MSCT perfusion parameters and MVD were analyzed. (III) The diagnostic value of CtBP2 and P16<sup>INK4A</sup> combined with MSCT in esophageal cancer.

### Statistical analysis

The data in this study were statistically analyzed using SPSS18.0 software. The measurement data were described by the mean  $\pm$  standard deviation. The t test was used to express the pass rate or composition ratio of the count data, and the  $\chi^2$  test was used. Correlation analysis was performed using Pearson's method, and a scatter plot was generated. The receiver operating characteristic (ROC) curve was used to analyze the diagnostic value of CtBP2 and P16<sup>INK4A</sup> combined with MSCT perfusion parameters and MVD in esophageal cancer. The difference was statistically significant at P<0.05and the test level was  $\alpha$ =0.05.

### **Results**

# Comparison of CtBP2 and P16^INK4A expression in the control group and the esophageal cancer group

As shown in *Table 1* and *Figure 1*, the positive expression rate of  $P16^{INK4A}$  in the esophageal cancer group was



Figure 1 CtBP2 and P16<sup>INK4A</sup> immunohistochemical detection (×200).

Table 2 Comparison of CtBP2 and P16<sup>INK4A</sup> expression in the control group and esophageal cancer group

Group	BF (mL·100 g <sup>-1</sup> ·min <sup>-1</sup> )	BV (mL/100 g)	MTT (s)	PS (mL·100 g <sup>-1</sup> ·min <sup>-1</sup> )	MVD
Esophageal cancer group (n=91)	92.69±29.02	5.42±1.66	8.62±3.25	11.06±4.14	23.85±6.05
Control group (n=42)	90.58±28.19	3.13±0.54	4.47±1.15	8.41±2.04	13.45±4.86
t	0.393	8.714	8.033	3.928	9.773
Р	0.695	<0.001	<0.001	<0.001	<0.001

CtBP2, C-terminal binding protein 2; P16<sup>INK4A</sup>, P16 inhibitor of cyclin-dependent kinase 4a; BF, blood flow; BV, blood volume; MTT, mean transit time; MVD, microvessel density; PS, permeability surface; P value, Probability; *t*-test, Student's t test.

significantly lower than that of the control group, while the positive expression rate of CtBP2 in the esophageal cancer group was higher than that of the control group (P<0.05).

# Comparison of CT perfusion parameter values and MVD in the control group and the esophageal cancer group

As shown in *Table 2*, the values of BV, MTT, PS, and MVD in the esophageal cancer group were increased compared with the control group (P<0.05). There was no significant difference in BF values between the 2 groups (P>0.05).

# Comparison of MSCT perfusion parameters and MVD in patients with different pathological characteristics

As shown in Table 3, the BF value of the tumor invading

the fibrous membrane was significantly higher than that of the tumor not invading the fibrous membrane (P<0.05). The PS and MVD values of patients with lymph node metastasis were higher than those without lymph node metastasis (P<0.05). There were no significant differences in different pathological types, degree of differentiation, and clinical stages for MSCT perfusion parameters and MVD (P>0.05).

# Correlation analysis of MSCT perfusion parameters and MVD

As shown in *Table 4* and *Figure 2*, MSCT perfusion parameters BF and BV were significantly positively correlated with MVD (P<0.05), while MTT, PS, and MVD were not significantly correlated with MVD (P>0.05).

Table .	3 Comparison of MSC1	□ perfusion parameter	s and MVD in pa	ttients with diffe	erent pathologic	al characteristics				
	Pathologic	al pattern	Degrees of di	fferentiation	Clinical	stages	Fibrous memb	rane invasion	Lymphatic	metastasis
LIOJEC	Squamouscarcinoma	a Adenocarcinoma	High-medium	Poorly	I-II stages	III stages	Yes	No	Yes	No
BF	92.85±29.65	88.81±25.16	90.33±28.43	92.53±29.31	91.33±29.04	92.57±29.17	108.82±52.22	72.36±47.77#	104.44±31.74	101.56±30.63
BV	5.52±1.63	5.11±1.26	5.73±1.51	5.52±1.69	6.24±2.23	6.72±2.84	6.38±1.74	6.06±1.52	6.34±3.02	6.89±2.11
ШШ	8.11±3.51	7.98±2.31	8.74±3.85	8.19±3.05	8.33±3.87	9.01±4.17	7.39±2.84	8.12±3.11	6.15±3.10	6.45±2.87
PS	11.14±4.82	12.04±5.01	13.19±6.92	12.44±6.24	13.08±4.47	13.99±4.52	9.18±3.68	10.33±4.01	17.12±5.29	8.55±3.58*
MVD	23.07±7.23	21.94±6.87	23.28±7.19	23.39±8.75	22.69±7.51	23.41±8.04	23.48±7.72	23.83±8.23	40.26±19.55	26.06±15.44*
Compe microv	tred with fibrous mem ssel density; PS, pern	brane invasion, <sup>#</sup> P<( meability surface; MS	0.05; compared SCT, multi-spiral	with lymph no CT; MVD, micr	de metastasis, ovessel density	*P<0.05. BF, b ; BF, blood flow	lood flow; BV, b v; BV, blood volur	lood volume; M ne; MTT, mean	TT, mean trans transit time; PS	it time; MVD, , permeability
surface	<i>.</i>									

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Table 4 Correlation analysis of MSCT perfusion parameters and MVD

MSCT parfusion parameter -		MVD
	r value	P value
BF	0.447	0.025
BV	0.567	0.003
MTT	-0.189	0.125
PS	0.201	0.094

MSCT, multi-spiral CT; MVD, microvessel density; BF, blood flow; BV, blood volume; MTT, mean transit time; PS, permeability surface. P value, Probability; *r* value, right value.

# The diagnostic value of CtBP2 and P16<sup>INK4A</sup> combined with MSCT in esophageal cancer

As shown in *Table 5* and *Figure 3*, ROC results showed that the areas under the curve (AUC) of CtBP2, P16<sup>INK4A</sup>, and MSCT were 0.625, 0.747, and 0.812, respectively. However, the area under the combined test curve was the largest, at 0.869.

### Case analysis

A 66-year-old male patient had progressive dysphagia for 3 months. Upper gastrointestinal angiography showed stenosis, stiffness, and mucosal disorder in the middle part of the esophagus. The upper esophagus was slightly dilated (*Figure 4A,B,C*). The CT plain scan showed that the middle part of the thoracic segment of the esophagus was thickened and the lumen was narrowed (*Figure 4D,E*), and the enhanced scan showed obvious continuous enhancement (*Figure 4F,G*). The pathology showed squamous cell carcinoma, moderate chronic inflammation of the gastric antrum mucosa, and mild acute activity (*Figure 4H,I*).

### Discussion

Esophageal cancer is a common malignant tumor of the digestive tract and has a high mortality rate. Due to the specific morphology of esophageal cancer, early detection is difficult, and most patients are in the middle and advanced stages of treatment (11). X-ray barium meal and CT examination are currently common methods for clinical diagnosis and efficacy evaluation. These examinations are limited due to their morbidity and indirect signs. Diagnosis still relies on endoscopic biopsy and ultrasound endoscopy, while MRI and radionuclide scanning only play



Figure 2 Correlation analysis.

Table 5 The diagnostic value of CtBP2 and P16<sup>INK4A</sup> combined with MSCT in esophageal cancer

Predictivefactor	Sensitivity	Specificity	AUC	95% CI
MSCT	0.718	0.694	0.812	0.688–0.936
CtBP2	0.535	0.723	0.625	0.463-0.787
P16 <sup>INK4A</sup>	0.668	0.736	0.747	0.615–0.879
MSCT + CtBP2 + P16 <sup>INK4A</sup>	0.952	0.885	0.869	0.766–0.972

CtBP2, C-terminal binding protein 2; P16<sup>INK4A</sup>, P16 inhibitor of cyclin-dependent kinase 4a; MSCT, multi-spiral CT; CI, confidence interval.



**Figure 3** Receiver operating characteristic (ROC) curve analysis of C-terminal binding protein 2 (CtBP2) and P16 inhibitor of cyclindependent kinase 4a (P16<sup>INK4A</sup>) combined with multi-spiral CT (MSCT) in esophageal cancer.

supplementary roles in its diagnosis. However, none of them can directly display the tumor blood supply and tumor angiogenesis (12).

CT perfusion imaging technology is a non-invasive functional imaging technique which can perform dynamic same-layer scanning of the lesion through contrast agent perfusion, obtain the time-density curve (TDC) of each pixel, and calculate the perfusion parameter values, such as BF, BV, MTT, PS, etc. (13). The blood perfusion status of the lesion can be directly evaluated based on the value of these perfusion parameters to reflect its development. At the same time, it can also judge the MVD and then evaluate the activity, pathological grade, and prognosis of the tumor (14). Tumor angiogenesis is the basic process of its growth and metastasis, and it is the physiological basis for tumor proliferation, nutrition supply, and metabolite elimination. Peripheral lung cancers with different histological types have different angiogenesis methods and numbers (15). In this study, statistical analysis found that there were no



**Figure 4** Case analysis. The patient, a 66-year-old male, had progressive dysphagia for 3 months. The upper gastrointestinal angiography showed that the middle part of the esophagus was narrow and rigid, and the mucous membrane was disordered. The upper esophagus was slightly dilated (A,B,C). CT plain scan showed local tube wall thickening and lumen stenosis in the middle of the thoracic segment of the esophagus (D,E), enhanced scan showed obvious continuous enhancement (F,G). Pathology showed: squamous cell carcinoma 22-27 cm from the incisors of the esophagus, moderate chronic inflammation of the gastric antral mucosa (H,I, HE staining,  $400\times$ ).

significant differences in CT perfusion parameters and MVD measurements of esophageal cancer with different differentiation levels and pathological types, which was consistent with some foreign literature reports. Tumor growth is mainly supported by new blood vessels with nutrients, which is the pathological basis for tumor cell metastasis and invasion, accounting for approximately 1-10% of the tumor volume (16). According to the definition, BF is mainly determined by the characteristics of tumor BF and the density of microvessels inside the tumor, which reflects the BF in the local area. BV changes mainly depend on the diameter of blood vessels, the number of open capillaries, and also the number of capillaries. It is generally believed that MVD is the gold standard for evaluating BV. The results of this study found that MVD is positively correlated with BF and BV, which is consistent with the report of Boothello et al. (17), further confirming the reliability of the above indicators for evaluating tumor angiogenesis.

Related foreign reports have found that molecular biology is also one of the more active research fields in addition to CT perfusion imaging, and it also plays an important role in the process of disease diagnosis and efficacy evaluation (18). P16<sup>INK4A</sup> is an important gene that regulates the cell cycle and inhibits cell division. This gene can also induce cell apoptosis and is inactivated in approximately 50% of human tumors, among which familial melanoma and cholangiomas are related to mutations in the P16<sup>INK4A</sup> gene (19). According to related reports, many other malignant tumors also have deletions and mutations of P16<sup>INK4A</sup> (20). Rajendra *et al.* (21) reported that P16<sup>INK4A</sup> was under-expressed in most esophageal cancer tissues, leading to cell cycle disorders and excessive cell proliferation. In this study, the positive expression rate of P16<sup>INK4A</sup> in esophageal cancer tissue was significantly lower than that in control group, which was consistent with the above-mentioned literature reports.

CtBP2 is an important member of the CtBP family and plays an important role in embryonic development, adipogenesis, and angiogenesis. In recent years, many studies have found that CtBP2 is closely related to tumors (22). In colon cancer, breast cancer, and prostate cancer, CtBP2 can promote tumor proliferation and cell migration through various signaling pathways. In this study, the positive expression rate of CtBP2 in the esophageal cancer group was significantly higher than that of normal esophageal mucosa, suggesting that CtBP2 may be involved in the occurrence and development of esophageal cancer. At present, a large number of foreign studies have shown that the sensitivity and specificity of a single test cannot be used to qualitatively diagnose malignant tumors. The main reason is that tumor cells and normal tissues can express certain genes (23). Studies have suggested that the combined detection of imaging and molecular biology can improve the diagnosis of malignant diseases, but there is no final conclusion (24). In order to effectively avoid the above-mentioned diagnosis limitations, clinicians often advocate for the combination of multiple methods to improve the accuracy of disease diagnosis. This group of studies combined CtBP2, P16<sup>INK4A</sup>, and MSCT for the diagnosis of esophageal cancer cases. ROC curve analysis results showed that the AUC of combined diagnosis was significantly higher than that of single detection, which suggested that the combination of CtBP2, P16<sup>INK4A</sup>, and MSCT could effectively improve esophageal disease diagnosis. The reason may be that MSCT, CtBP2, and P16<sup>INK4A</sup> can provide a dual basis for imaging and biochemical indicators for the clinical diagnosis of esophageal cancer, which makes the diagnosis evidence more sufficient, and makes up for the deficiency of single diagnosis and improves the accuracy of clinical diagnosis. However, this study has not yet compared the combined diagnostic value of the 2 indicators, and expansion of the sample size is needed for further discussion. In summary, MSCT perfusion imaging of esophageal cancer lesions can indirectly reflect the angiogenesis of esophageal cancer in vivo, and the combination of CtBP2 and P16<sup>INK4A</sup> can effectively improve the diagnostic efficiency of the disease.

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All patients agreed to participate in this study and signed an informed consent form. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Henan Provincial Chest Hospital (No. 20181224).

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