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Correlations of Arterial Phase CT Characteristics of Hepatocellular Carcinoma with Differentiation Degree and Expressions of PCNA and PTEN

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ABSTRACT The purpose of this study was to explore the correlations of the arterial-phase enhanced CT characteristics of hepatocellular carcinoma (HCC) with the differentiation degree and expressions of proliferating cell nuclear antigen (PCNA) and phosphatase and tensin homologue (PTEN). Seventy-nine HCC patients who underwent three-phase dynamic contrast-enhanced CT before surgery were selected to observe the arterial-phase characteristics. The degree of HCC tissue differentiation was evaluated by hematoxylin-eosin staining. PCNA and PTEN expressions were detected by immunohistochemistry. Spearman's correlation analysis was conducted. The tumour size and vascular enhancement characteristics were negatively correlated with HCC differentiation degree and PTEN expression, and positively correlated with PCNA expression. The capsule integrity was positively correlated with HCC differentiation degree and PTEN expression, and negatively correlated with PCNA expression. The arterial-phase CT characteristics of HCC reflect the differentiation degree and expressions of PCNA and PTEN.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most lethal cancers due to poor prognosis, high mortality rate and easy recurrence (Giannelli et al. 2016). Pathological examination is the golden standard for the diagnosis of HCC, with immunohistochemistry as an important auxiliary method (Woo et al. 2022). Recently, p53 and XRCC1 genotypes have been detected to diagnose HCC (Devi et al. 2013), and serum AFP level has been measured to determine the prognosis (Devi et al. 2010). Although these markers can well reflect the onset of HCC, they cannot provide the real-time tumour growth *in vivo* because specimens are usually collected by biopsy or surgical resection. Additionally, it is difficult to repeat examinations during follow-up (Balachandar and Sasikala 2013). Spiral CT, as one of the commonly used imaging methods for HCC, allows a wide range of thin-slice scans in a short time, with clear three-dimensional reconstruction results (Chen et al. 2019b). This method provides different enhancement characteristics of tumour tissues and their adjacent normal tissues at various stages (Zhao et al. 2017). Recently, the detection rate and accuracy of HCC have been elevated significantly (Kim et al. 2017). Since the imaging manifestations of HCC are based on the changes of histopathology and morphology determined by biological behaviours, it is of great significance to explore the relationship between imaging results and molecular biology for clinical diagnosis and prognosis evaluation (Reginelli et al. 2018). Proliferating cell nuclear antigen (PCNA), as a tumour marker closely related to cell proliferation and cycle, is one of the important indices for assessing the tumour proliferation ability and malignancy degree (de Oliveira et al. 2017). Besides, phosphatase and tensin homologue (PTEN) is a tumour suppressor gene that plays a crucial role in maintaining the balance between cell proliferation, differentiation and apoptosis (Li et al. 2017), and its positive expression rate is negatively correlated with the malignancy degree of HCC (Chen et al. 2019a).

Objectives

In this study, the Spearman's correlation analysis was performed to clarify the correlations of the arterial-phase CT characteristics with the degree of HCC differentiation as well as the expressions of PCNA and PTEN, aiming to provide valuable clinical evidence.

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MATERIAL AND METHODS

Materials

Seventy-nine HCC patients who were surgically resected and pathologically confirmed in the researchers' hospital from January 2015 to December 2017 were selected. There were 43 males and 16 females who were aged from 28 to 73 years old, with an average of 52.3 years. All patients had primary HCC and underwent three-phase dynamic contrast-enhanced CT before surgery, without receiving any intervention or radiofrequency treatment. This study was approved by the ethics committee of the hospital, and the patients and their families voluntarily signed the informed consents.

Anti-PCNA antibody (Cat. No. ab92552) and anti-PTEN antibody (Cat. No. ab267787) were purchased from Abcam (USA). Hematoxylin-eosin (HE) staining kit (Cat. No. C0105S) was bought from Beyotime Biotechnology Co., Ltd. (China).

CT Scan Method

The process for the CT scan was used for the patients. The Siemens Definition AS 128-slice helical CT scanner (Germany) was used. The patients were required to fast for 5 to 6 hours before examination, and to drink 1000 ml of water 20 minutes before examination and another 200-300 ml again before lying on the examination bed to fill the stomach and intestines and to improve tissue contrast. A plain CT scan was performed by crossing the hands on the top of the head and setting the scan range from the diaphragm to the lower edge of the liver. The direction of bed entry was from head to toe. The scan parameters were set as pitch of 0.8, collimator of 0.6 mm and tube voltage of 120 kV. The CARE Dose4D system was used to automatically adjust the tube current according to the patient's body size without affecting the diagnosis. Contrast agent Ultravist (300 mgI/ml, Bayer HealthCare Company Limited Guangzhou Branch, China) was injected at 1.5 to 2.0 ml/kg body weight for imaging. The injection rate was 3 to 3.5 ml/s. The agent was injected into the antecubital vein by a specific high-pressure syringe through an 18G indwelling needle. Afterwards, 20 to 50 ml of normal saline was injected for flushing. After injection of contrast agent, dynamic tracking was conducted to set a region of interest in the abdominal aorta to detect CT value. When the value reached the threshold (100 HU), the arterial-phase scan was triggered. The portal venous and equilibrium phases were 65 seconds and 120 seconds after injection, respectively. Lastly, for image reconstruction, after examination, the raw data were reconstructed with the slice thickness of 5 mm, the convolution kernel of B30f medium smooth and FOV of 512×512.

CT Observation Indices

Imaging results were evaluated by three radiologists in a blinded manner, including the lesion location, morphology, tumour size, capsules and enhancement characteristics. The tumour size (diameter $\leq$5.0 cm and >5.0 cm), capsule condition (complete, incomplete or no capsule) and arterial-phase enhancement characteristics (parenchymal, vascular or unobvious enhancement) were selected as observation indices.

Observation of HCC Tissue Morphology by HE Staining

After being soaked in paraformaldehyde for 48 hours, HCC tissues were dehydrated, embedded in paraffin and cut into 4 μm-thick sections, followed by deparaffinization, hydration, HE staining, dehydration, sufficient immersion in xylene for 2 hours, transparentization and drying. The sections were mounted with resin, and the tissue morphology was observed and photographed under an optical microscope. According to the histological classification criteria of hepatic and intrahepatic cholangiocarcinoma by WHO (Nakanuma et al. 2010), the selected cases were divided into high differentiation group (highly or highly-moderately differentiated), moderate differentiation group (moderately or moderately-lowly differentiated), and low differentiation group (lowly differentiated or undifferentiated).

Detection of PCNA and PTEN Expressions in HCC Tissue by Immunohistochemistry

The tissue sections were routinely deparaffinized, hydrated, placed in PBS for high-pressure retrieval, soaked in three percent hydrogen peroxide at room temperature to block endogenous peroxidase, blocked, incubated with diluted PCNA and PTEN antibodies (Abcam, USA) at 4°C overnight,

washed with PBS after rewarming on the next day, incubated with diluted secondary antibodies at room temperature for 30 minutes, washed with PBS and incubated with an appropriate amount of DAB for 5 minutes. After the reaction was stopped by adding deionised water, the sections were counterstained with hematoxylin for 5 minutes, rinsed with sterile water and dehydrated for 2 hours with gradient concentrations of ethanol solutions. After the residual liquid on the surface was absorbed with sterile filter paper, xylene was used for transparentization, and the sections were mounted with resin. The tissue morphology was observed and photographed under the optical microscope. The proportion of positive cells to cancer cells was calculated, with less than thirty percent as the negative group and greater than or equal to thirty percent as the positive group.

Statistical Analysis

All data were statistically analysed using SPSS 19.0 software. Intergroup comparisons were performed by the χ^2 test. Correlations were studied by Spearman's analysis. $P<0.05$ was considered statistically significant.

RESULTS

Pathological Morphology of HCC and Normal Tissues

Of the 79 HCC tissue samples, 18 had moderate differentiation degree, 36 and moderate differentiation degree and 25 had low differentiation degree. The cells in the highly differentiated HCC group began to show low heterogeneity. With decreasing degree of differentiation, the heterogeneity gradually increased, resulting in the loss of inherent tissue structure, disorder of cell arrangement and enhancement of nuclear division (Fig. 1).

PCNA and PTEN Expressions in HCC and Normal Tissues

PCNA was located in the nucleus, and brownish yellow particles indicated positive expression. The overall positive expression rate of PCNA in HCC tissues was 64.6 percent, and the rates of high, medium and low differentiation groups were 33.3 percent, 72.2 percent and 76.0 percent, respectively. High PCNA expression reflects strong cancer cell proliferation, indicating that the proliferation ability was enhanced in patients with lowly differentiated HCC. PTEN was located in the cytoplasm, and brownish yellow particles suggested positive expression. The overall positive expression rate of PTEN in HCC tissues was 30.4 percent, and the rates of high, medium and low differentiation groups were 55.5 percent, 25.0 percent and 20.0 percent, respectively (Fig. 2 and Table 1). Therefore, the positive expression rate of PCNA significantly increased but that of PTEN decreased with reducing HCC differentiation ($P<0.05$).

Correlations Between Arterial-phase CT Characteristics and Differentiation Degree of HCC

The arterial-phase CT characteristics are shown in Figure 3 and Table 2. The proportion of high differentiation in the tumour $\leq$3.0 cm group was higher than that of the tumour >3.0 cm group, the proportion of the group with intact capsule was higher than that of the group with incomplete or no capsule, and the proportion of the group without obvious enhancement was higher than that of the enhancement group ($P<0.05$). Spearman's correlation analysis showed that the degree of HCC differentiation was negatively correlated with tumour size and enhancement characteristics, but positively correlated with the capsule integrity. In

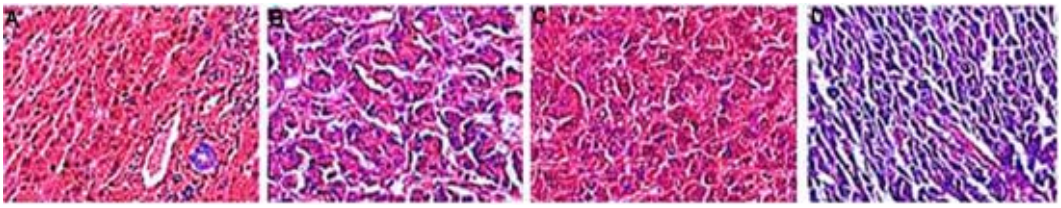

Fig. 1. Pathological morphology of HCC and normal tissues. A: Normal tissue; B-D: Highly, moderately and lowly differentiated HCC tissues. HCC: Hepatocellular carcinoma

Table 1: PCNA and PTEN expressions in HCC and normal tissues

Differentiation degree	*No. of cases*	*PCNA*		χ^2	*P*	*PTEN*		χ^2	*P*
		Positive	*Negative*			*Positive*	*Negative*		
High	18	6	12	10.025	0.007	10	8	7.160	0.028
Moderate	36	26	10			9	27		
Low	25	19	6			5	20		
Total	79	51	28			24	55		

HCC: Hepatocellular carcinoma; PCNA: proliferating cell nuclear antigen; PTEN: phosphatase and tensin homologue

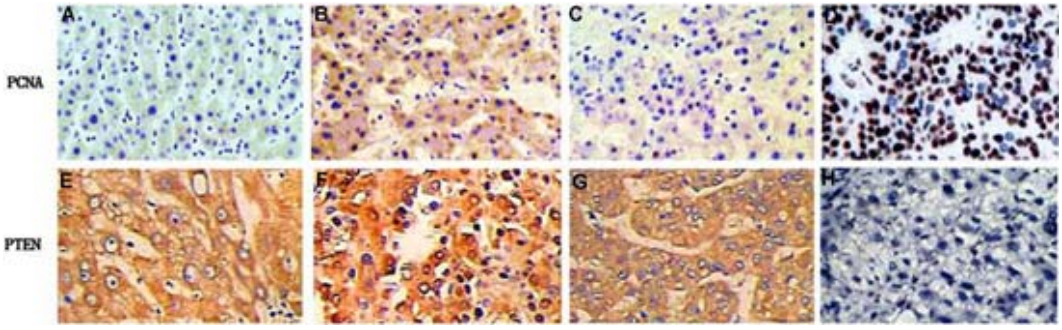

Fig. 2. PCNA and PTEN expressions in HCC and normal tissues. A and E: Normal tissue; B and F: highly differentiated HCC tissue; C and G: moderately differentiated HCC tissue; D and H: lowly differentiated HCC tissue. HCC: Hepatocellular carcinoma; PCNA: proliferating cell nuclear antigen; PTEN: phosphatase and tensin homologue

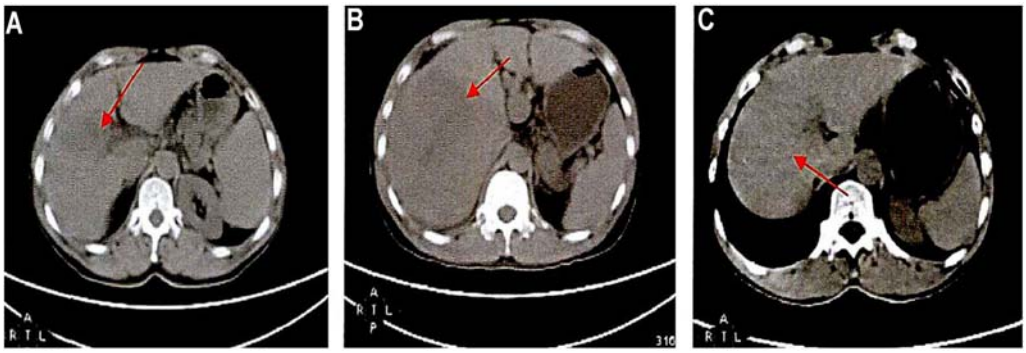

Fig. 3. Arterial-phase CT characteristics of HCC tissues with different differentiation degrees. A: A female patient, 62 years old, had no obvious enhanced tumor blood vessels, suggesting that tumors was well differentiated, and the pathological findings showed highly-differentiated HCC; B: a male patient, 47 years old, with visible tumor blood vessels, pathologically demonstrated moderately differentiated HCC; C: in a 54-year-old male patient, the tumor at the arterial phase showed mass and patchy enhancement, with poor tumor differentiation, and the pathology showed lowly differentiated HCC. HCC: Hepatocellular carcinoma

other words, a smaller tumour had a more complete capsule, less obvious characteristics of vascular enhancement, higher degree of differentiation and lower degree of malignancy. Thus, the characteristics of CT arterial phase imaging can reflect the degree of HCC differentiation.

Table 2: Correlations between arterial-phase CT characteristics and differentiation degree of HCC

Imaging characteristics		*No. of cases*	*Differentiation degree*			χ^2	P_1	*R*	P_2
			High	*Moderate*	*Low*				
Tumor size	≤3.0 cm	37	13	16	8	6.9514	0.031	-0.283	0.011
	>3.0 cm	42	5	20	17				
Capsule	Complete	30	12	17	4	12.724	0.002	0.385	0.000
	Incomplete or no	49	6	19	21				
Enhancement characteristics	Unobvious enhancement	33	13	14	6	10.231	0.006	-0.344	0.002
	Enhancement	46	5	22	19				

HCC: Hepatocellular carcinoma

Correlations of Arterial-phase CT Characteristics with PCNA and PTEN Expressions

The positive expression rate of PCNA in the tumour ≤3.0 cm group (17/37) was significantly lower than that of the tumour >3.0 cm group (34/42), and the rate of the group with intact capsule (13/30) was lower than that of the group with incomplete or no capsule (38/49), and the rate of the group without obvious enhancement (15/33) was lower than that of the enhancement group (36/46) (P<0.05). The positive expression rate of PTEN in the tumour ≤3.0 cm group (17/37) was significantly higher than that of the tumour >3.0 cm group (7/42), and the rate of the group with intact capsule (16/30) was higher than that of the group with incomplete or no capsule (8/49), and the rate of the group without obvious enhancement (18/33) was higher than that of the enhancement group (6/46) (P<0.05). It was further found by Spearman's correlation analysis that the expression of PCNA was positively correlated with tumour size and enhancement characteristics, and negatively correlated with the conditions of the capsule (P<0.05). The expression of PTEN was negatively correlated with tumour size and enhancement characteristics, and positively correlated with the conditions of the capsule (P<0.05) (Tables 3 and 4). Accordingly, CT arterial imaging characteristics can reflect the expression levels of PCNA and PTEN.

DISCUSSION

Due to the high degree of malignancy and poor prognosis of HCC, early detection, diagnosis and treatment play key roles in improving the treatment outcomes (Shimoda et al. 2018). It is of great significance for the early diagnosis, treatment and prognosis of HCC to find tumour markers with high diagnostic accuracy and to evaluate them by CT images. CT examination is currently widely used in the diagnosis, follow-up and treatment outcome assessment of HCC (Krishan and Mittal 2022). It has the advantages of non-invasiveness, easy operation and high repeatability. The accurate pre-

Table 3: Correlations of arterial-phase CT characteristics with PCNA expression

Imaging characteristics		*No. of cases*	*PCNA*		χ^2	P_1	*R*	P_2
			Positive	*Negative*				
Tumor size	≤3.0 cm	37	17	20	10.535	0.001	0.365	0.001
	>3.0 cm	42	34	8				
Capsule	Complete	30	13	17	9.522	0.002	-0.347	0.002
	Incomplete or no	49	38	11				
Enhancement characteristics	Unobvious enhancement	33	15	18	9.038	0.003	0.338	0.002
	Enhancement	46	36	10				

PCNA: Proliferating cell nuclear antigen.

Table 4: Correlations of arterial-phase CT characteristics with PTEN expression

Imaging characteristics		*No. of cases*	*PCNA*		χ^2	P_1	*R*	P_2
			Positive	*Negative*				
Tumor size	?3.0 cm	37	17	20	7.973	0.005	-0.318	0.004
	>3.0 cm	42	7	35				
Capsule	Complete	30	16	14	12.049	0.001	0.391	0.000
	Incomplete or no	49	8	41				
Enhancement characteristics	Unobvious enhancement	33	18	15	16.648	0.000	-0.445	0.000
	Enhancement	46	6	40				

PTEN: Phosphatase and tensin homologue

liminary evaluation of HCC patients using CT images is of great significance for further examination, treatment and prognostic evaluation (Yoon et al. 2020).

It is generally accepted that lower degree of tumour differentiation represents higher degree of malignancy (Shao et al. 2017). In CT images, the capsule exists as a circular shadow on the edge of the tumour, and its integrity can indicate the differentiation of HCC (Wang et al. 2018). In the early stage, HCC hardly infiltrates adjacent tissues, and has a clear margin and intact capsule, while in the late stage, HCC shows infiltrative growth and is difficult to form fibrous capsules (Chuaypen et al. 2018). As the malignancy degree of HCC increases, the number of abnormal tumour arteries increases, and the enhancement of CT arterial phase can reflect tumour hemodynamic changes (Yang et al. 2016). Highly differentiated HCC is not obviously enhanced at the arterial phase, while lowly differentiated HCC has obvious enhancement at the arterial phase (Rhee et al. 2019). In this study, the Spearman's correlation analysis revealed that the degree of HCC differentiation was positively correlated with the integrity of capsule, and negatively correlated with the size and enhancement characteristics of the tumour, indicating that the enhancement characteristics of arterial phase CT can reflect the degree of HCC differentiation.

PCNA is a marker related to cell cycle and proliferation, which participates in the DNA synthesis of cancer cells (Ho et al. 2017). Its expression is related to tumour histological grade. The high expression of PCNA reflects strong proliferation of cancer cells, especially in malignant tumours with poor tissue differentiation such as HCC (Kitamura et al. 2019). PCNA can be used as a potential diagnostic biomarker for early HCC (Ma et al. 2016). The results herein showed that PCNA expression increased with decreasing HCC differentiation degree. The positive expression rate of PCNA was negatively correlated with the integrity of the capsule, and positively correlated with the tumour size and enhancement characteristics. Collectively, the arterial phase CT characteristics can reflect the expression level of PCNA.

As a tumour suppressor gene, PTEN has attracted much attention in tumour research, which can inhibit the adhesion of cancer cells and the infiltration and metastasis to surrounding tissues through dephosphorylation, and also inhibit the cycle of cancer cells and promote their apoptosis (Huang et al. 2017). PTEN is mutated in a variety of tumours, including HCC (Akula et al. 2019). In addition, hypermethylation of the promoter region and gene silencing caused by miRNA can also cause the loss of PTEN function (Lu et al. 2017). About forty percent of HCC cases have PTEN deficiency or expression reduction, which is related to low differentiation, poor prognosis and recurrence (Xu et al. 2018). This study showed that the expression of PTEN decreased significantly with reducing HCC differentiation degree. Szparecki et al. (2017) also found that PTEN expression was significantly absent in lowly differentiated HCC, but its expression in highly and moderately differentiated HCC was similar to that in normal tissues. Herein, the expression of PTEN was positively correlated with the integrity of the capsule, and negatively correlated with the size and enhancement characteristics of the tumor. Taken together, CT image characteristics can reflect the expression level of PTEN.

CONCLUSION

In summary, the expressions of PCNA and PTEN can reflect the differentiation degree of HCC, and both can be evaluated by the characteristics of CT arterial phase enhancement.

RECOMMENDATIONS

It is recommended to observe the characteristics of CT arterial phase enhancement, which provides valuable evidence for the clinical selection of treatment methods and prognostic evaluation of HCC patients.

ABBREVIATIONS

HCC: hepatocellular carcinoma
HE: hematoxylin-eosin
PCNA: proliferating cell nuclear antigen
PTEN: phosphatase and tensin homologue

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DECLARATION OF INTEREST

The author declares no competing interest.

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