

Sinusoidal endothelial cell progenitor cells promote tumor progression in the patients with hepatocellular carcinoma.

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Research

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Abstract

Objective: Since sinusoidal endothelial cell progenitor cells (SEPCs) play great roles in liver regeneration, it is necessary to elucidate that whether these SEPCs participate in tumor progression of hepatocellular carcinoma (HCC).

Methods: A total of 45 patients with primary HCC, who received liver resection, were included in this study. The liver tumors were removed from patients and partial tissues were prepared to identify SEPCs by double staining of CD133/CD45 and CD133/CD31 at the same location. Blood samples were correspondingly collected to examine liver function parameters and tumor markers. The demographics and clinic-pathological characteristics of the patients were all collected for correlation analysis with SEPCs.

Results: SEPCs were observed within abundant of blood vessels in HCC nodules of all the 45 patients, but no SEPCs were detected in the tumor-adjacent tissues. The number of SEPCs was correlated with the expression levels of HCC tumor markers α -fetoprotein (AFP) and CA199. There was a positive correlation between the expressions of SEPCs markers and the diameters of HCC tumors in differently differentiated specimens ($P < 0.01$). The expressions levels of SEPCs markers in poorly differentiated HCC patients were significantly higher than those in the moderately and highly differentiated HCC patients ($P < 0.05$).

Conclusions: SEPCs are in close connection with HCC progression, and they may potentially act as prognosis and metastasis predicting index and even therapeutic candidates of HCC.

Introduction

Hepatocellular carcinoma (HCC) was the sixth most prevalent cancer in the world, which was the main cause of cancerous mortalities ¹. The high incidence of HCC in Asia and Sub-Saharan Africa resulted from the high prevalence of infection to the patients with hepatitis B virus (HBV) ^{2,3}. Although surgical operation, radiotherapy, and immunotherapy were applied for treating HCC, the curative effect of advanced HCC was still unsatisfied owing to the side effects, drug-resistance and recurrence ^{4,5}. Therefore, it is necessary to elucidate the molecular mechanisms of HCC tumorigenicity and progression in order to get early diagnosis, make right therapeutic strategies, and prevent the HCC becoming worse. By far, considerable studies put accumulated focus on the tumor cells and related signal pathways. Few studies have explored the effect of peri-tumor angiogenesis on the malignant tumor formation and progression.

Angiogenesis was vitally important to malignant tumor formation, progression and metastasis. Proliferation and migration of endothelial cells took part in the process of angiogenesis, followed by the formation of new capillaries ⁶. The hypoxic tumor microenvironment promoted excessive secretion of pro-angiogenic factors from neoplastic, stromal and infiltrating immunocytes. Excessively pro-angiogenic factors stimulated abnormal angiogenesis, such as blood vessels with structural abnormalities. Once

tumor blood vessels formed, they provided critical oxygen and nutrients to satisfy highly-demanding metabolic requirements and support rapid tumor growth. The abnormally structural and functional tumor vessels facilitated hematogenous metastasis, a hallmark of aggressive malignancies associated with poorly survival rates⁷. Furthermore, these defective vessels were poorly perfused, limiting drug delivery to tumorous regions and resulting in reduced efficacy of anti-cancer agents. Targeting therapy of vascular endothelial growth factor (VEGF) was one of the most common therapeutic strategies against tumor angiogenesis⁸.

Since angiogenesis required a large number of sinusoidal endothelial cells, sinusoidal endothelial cell progenitor cells (SEPCs), as the progenitor of sinusoidal endothelial cells, were found to be the seed cells of angiogenesis^{9,10}. The SEPCs also have an important role in regulating the secretion of hepatocyte growth factor (HGF). The SEPCs were triple positive for the progenitor cell marker CD133, the endothelial cell marker CD31 and the hematopoietic cell marker CD45 as well. Numerous of studies regarding liver regeneration have indicated that bone marrow (BM)-derived sinusoidal endothelial cell progenitor cells (BM SEPCs) drove the liver regeneration process. These BM SEPCs repaired lost or injured liver sinusoidal endothelial cells (LSECs), leading to engraftment of a substantial number of SEPCs and BM-derived LSECs through signaling by VEGF-stromal cell derived factor 1 (sdf1) after injury or partial hepatectomy¹¹⁻¹⁵. The VEGF also stimulated increased HGF expression in the cells of the LSEC fraction, i.e., mature LSECs and/or SEPCs¹².

Since VEGF has been widely proved to contribute to HCC progression and VEGF regulated several key steps of SEPCs activation in liver repairment, it is necessary to investigate the contributing roles of SEPCs during HCC development. Herein, we are aimed to explore the potential relationship between SEPCs and HCC progression.

Patients And Methods

1.1 Patients and samples

Total 45 patients with histologically confirmed primary HCC, who underwent surgical resection at the Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital between January 2017 and December 2019, were consecutively enrolled into the present study. All patients did not have other malignancies or a history of drinking for at least 5 years. In addition, these patients were diagnosed with the disease for the first time and did not receive radiotherapy, chemotherapy, or immunotherapy. Patients were assessed by clinical examination, laboratory parameters, ultrasound, CT scans and MRI imaging.

Blood and tissue samples and patient data were provided by Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital. All samples were fully anonymized except for sex and age. Written informed consent was obtained from all patients and the study was approved by the institutional review board of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital.

This study was performed in accordance with clinical study protocols and the principles of the Declaration of Helsinki (modified 2000) and approved by the Research Care and Ethics Committee of our institution (No. SPPHCT2017–MED-10).

1.2 Clinic features

Clinic features were obtained from the enrolled patients, including demographic characteristics (age, gender, BMI, ethnic group, history of smoking, and history of drinking), and clinicopathologic features (HBV surface antigen, pathological grade, metastasis), laboratory parameters (blood routine test, total protein, albumin, total bilirubin, direct bilirubin, indirect bilirubin, ALT, and AST), tumor makers (AFP, CEA, CA-199, and CA-125), and tumor diameter (the largest diameter of tumor).

1.3 Blood sampling

The blood samples from 45 patients with HCC were centrifuged at 1500g/min for 10 min at 4°C, followed by the speed of centrifugation 2000g/min for 3 min at 4°C. Then the serous samples were stored at -80°C. Routine laboratory parameters were collected at the Department of Clinical Laboratory in the Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital.

1.4 Hematoxylin-eosin (HE) staining and Immunofluorescence staining

A total of 45 pairs of tumorous and adjacently nontumorous liver tissues were collected from surgically resected HCC specimens. Samples were then formaldehyde-fixed and paraffin-embedded, sectioned to 5 µm slices. Slices of 45 tumorous liver tissues were de-waxed in xylene followed by HE staining and microscopic observation, so as to determine the differentiation grades of HCC. Immunohistochemistry staining scores of HCC differentiation in liver tissues were assessed using a semi-quantitative method by two pathologists who did not have knowledge of the clinical data previously. Immunofluorescence staining was carried out on the slices of 45 pairs of tumorous and adjacently nontumorous liver tissues. Via immunofluorescence staining test, endogenous avidin and biotin were blocked by the avidin-biotin kit (Vector Labs) following the protocol. The primary antibodies of anti-CD133 (cat.:BF0403 Affinity Biosciences. OH.USA), anti-CD45 (cat.: DF2912 Affinity Biosciences. OH.USA), and anti-CD31 (cat.:AF6191 Affinity Biosciences. OH.USA) were used in this study. The primary antibody was recognized by the fluorescent secondary antibody and visualized by Fluorescence Inverse Microscope (Olympus X81). Four views of each section were randomly recorded, with three sections obtained from one sample. The positive staining signals in HCC tissues were quantitated by Image-Pro Plus software. Pictures were observed by Olympus microscope in 10×10 magnification. The target specimens were double stained of CD133/CD45 and CD133/CD31.

1.5 Statistical analysis

Continuous variables were shown by means ± standard deviation and categorical variables were reported as frequencies and percentages. Differences in the expression levels of SEPCs markers between different patient cohorts were determined by using ANOVA tests. *P* values < 0.05 were considered to be significant.

Statistical analyses were performed with SPSS (Version 22.0, IBM, New York, USA). Figures were made by GraphPad Prism version 6.0 (GraphPad Software, San Diego, California, USA).

Results

2.1 Patients demographic characteristics and histopathology

A total of 45 HCC patients were reviewed at Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital from January 2017 to December 2019, of which, 45 patients with primary HCC met inclusion criteria. The demographics and clinic-pathological parameters of the enrolled 45 patients including age, gender, ethnic group, pathological grade, metastasis, presence of HBV were shown in Table 1. All the 45 patients were Han nationality people diagnosed with primary HCC by pathology for the first time and did not have other malignancies. The proportion of above 50 years and male in patients was 55.6% and 91.1%, respectively.

The HE staining results suggested that differently differentiated HCC lesions were detected in 45 patients. The typical cases of HCC with poor differentiation, moderate differentiation and high differentiation are shown in Figure 1. In highly differentiated HCC, most of the cancer cells with increased nucleocytoplasmic ratio were arranged trabecularly and accompanied by alveolar structure, fat changes could be seen as well (Figure 1A). In moderately differentiated HCC, cells rich in cytoplasm and had clear nucleolus were arranged into small trabecular structure or cell cord (Figure 1B). In poorly differentiated HCC, the nucleocytoplasmic ratio of cancer cells increased significantly, and the tumor cells were with obvious pleomorphism, varied size and distinct shape (Figure 1C).

Additionally, the majority (86.7%, 39/45) of HCC patients were HBV positive. For pathological grade, high differentiation, moderate differentiation and low differentiation accounted for 20% (9/45), 20% (9/45) and 60% (27/45), respectively. Among these patients, 11 patients (24.4%) did not occur metastases, 34 patients (75.6%) suffered from metastases. The focal liver lesions located at right lobe liver or left three lobe liver. Correspondingly, these 45 patients received right hepatectomy or left trisegmentectomy according to the focal liver lesion location and did not receive radiotherapy, chemotherapy, or immunotherapy.

2.2 Localization of SEPCs in HCC patients

The SEPCs with CD31, CD45 and CD133 expressions was consider as progenitor cells of LSECs, therefore, we used double staining of CD133/CD45 and CD133/CD31 at the same location to prove that the staining was triple positive so as to determine phenotypic signature for SEPCs. The SEPCs were found within abundant of blood vessels in tumor nodules from all 45 patients, and we didn't observe any SEPC in the tumor-adjacent tissues (Figure 2). SEPCs were significantly more in tumorous than in adjacent nontumorous liver tissues ($P < 0.01$) (Figure 2).

2.3 Liver functional data

As shown in Table 2, liver functional data including total protein, albumin, direct bilirubin, indirect bilirubin, ALT and AST. Total protein, albumin, direct bilirubin, indirect bilirubin and ALT were normal in most cases, and AST exhibited approximately equal ratio of high and normal cases (23 versus 22 cases). A linear regression analysis revealed there was no correlation between SEPCs expressions and total protein, albumin, direct bilirubin, indirect bilirubin, ALT and AST levels (Figure 3).

2.4 Tumor markers

As shown in Table 3, HCC tumor markers including AFP, CA199, CEA and CA125 were tested. Most patients were with high levels of AFP, while CA199, CEA and CA125 were normal in most HCC patients. Furthermore, linear regression analysis indicated there was a strong positive correlation between SEPCs markers' expression and AFP or CA199 levels (Figure 4A, B). But this was no correlation between SEPCs markers' expression and CEA and CA125 level (Figure 4C, D).

2.5 The relationship between SEPCs and the size of HCC

The diameter of HCC tumors (the largest diameter of tumor) in highly differentiated, moderately differentiated, and poorly differentiated specimens were measured respectively to explore the relationship between SEPCs and the size of HCC lesion in the different differentiated cases. According to the diameter of tumors measured, samples were divided into three groups, i.e. 1.5-5 cm group, 5-9 cm group and 9-16 cm group. Then, we observed the SEPCs contents in each HCC specimen (Figure 5 A, B). The linear regression analysis revealed there was a positively strong correlation between SEPCs contents and HCC tumor diameter in highly differentiated, moderately differentiated, and poorly differentiated specimens, respectively ($P<0.01$) (Figure 5 C).

2.6 The relationship between SEPCs contents and the differentiation grades of HCC

In order to explore the relationship between SEPCs and the differentiation of HCC, we observed the SEPCs contents in highly differentiated, moderately differentiated, and poorly differentiated specimens. The SEPCs contents in poorly differentiated HCC patients were significantly higher than those in the moderately and highly differentiated HCC patients ($P<0.01$) (Figure 6B). Meanwhile, compared with highly differentiated HCC, the expression of SEPCs in moderately differentiated HCC was also statistically different ($P<0.05$).

Discussion

HCC was the third cause of cancer-related mortality worldwide, which mainly due to advanced stage of HCC at the time of initial diagnosis¹⁶. Its high rate of recurrence and resistance after conventional therapy was caused by high angiogenic and metastatic potential of HCC cells, and the rate of recurrence was as high as 70% following conventional treatments, such as chemotherapy, arterial embolization, surgical resection, and radiofrequency ablation¹⁷⁻¹⁹. Present studies mainly focused on the HCC cancer cells, such as the metabolism of cancer cells^{18,20} and high degree of molecular heterogeneity in liver

tumors²¹. But, few studies have explored the effect of peri-tumor environment such as angiogenesis on the malignant tumor formation and progression.

Angiogenesis, an important feature of cancer, was induced early during the multistage development of cancers^{22,23}. High levels of angiogenesis related factors are significantly associated with rapid cancer recurrence and poorly survival^{24,25}. Blocking synthesis and secretion of these growth factors would greatly impair tumor cell proliferation, metastasis and angiogenesis²⁶⁻²⁹. However, the underlying mechanisms were not fully understood, and few studies have been conducted to explore the effect of peri-tumor angiogenesis and the vessel released factors on the tumor diagnosis, formation, development and metastasis. Hence, the discovery of novel diagnostic and therapeutic targets on angiogenesis is crucial for the patients with HCC.

A small amount of SEPCs were the progenitors of LSECs that exist in normal liver tissue, expressing CD31, CD45 and CD133. Previous studies have indicated that BM SEPCs drove the liver regeneration process. These BM SEPCs repaired lost or injured LSECs, leading to engraftment of a substantial number of BM SEPCs and BM-derived LSECs after injury or partial hepatectomy^{11,13}. Recruitment of BM SEPCs to the liver occurred through activated signaling of VEGF- sdf1 and BM SEPCs provided most of the increase in LSEC-related HGF after injury, which further promoted hepatocyte proliferation and normal liver regeneration^{8,12}. Previous studies indicated that, hepatic VEGF regulated recruitment of BM-SEPCs to injured liver to help repair damage¹², and the influx of BM-SEPCs provided most of the increase in HGF after injury and promotes hepatocyte proliferation and normal liver regeneration³⁰. However, no studies have been conducted to explore whether SEPCs were related to HCC progression. Herein, this study revealed the presence of SEPCs in HCC and these SEPCs could be detected in vascular-rich areas of the tumor tissues. In addition, we also demonstrated that SEPCs contents were related to the expressions of AFP, tumor sizes and differentiation grades of HCC. Together with previous studies, we speculated that SEPCs might be crucial in the progression of HCC through factors secretion and tumor angiogenesis effects.

HCC is difficult to diagnose early, to treat promptly and effectively due to the lack of typical symptoms at early stage. At present, AFP is the most common serological test used for screening and diagnosis of HCC, as well as for surveillance after treatment. However, there were serious limitations with AFP, such as low sensitivity³¹, false-negatives and false-positives owing to conditions such as pregnancy and certain gastrointestinal tumors^{32,33}. From this study, SEPCs levels were demonstrated a positive correlation with AFP level, which indicated that SEPCs could be a potential target in HCC diagnosis by acting as a complementary tumor marker. Comparison between AFP and SEPC in the diagnosis of liver cancer, with pathological diagnosis as the gold standard, needs to be conducted in future to explore whether SEPCs have a better effect than AFP in the HCC. Furthermore, future studies could investigate the potential roles of factors secreted by SEPCs as the complementary tumor markers in diagnosis.

In our study, we also explored the relationship between SEPCs and the size of HCC. As the tumor size varied greatly even in the same differentiation grade, we observed the diameter of HCC tumors (the largest diameter of tumor) in highly differentiated, moderately differentiated, and poorly differentiated specimens, separately. The linear regression analysis revealed that there was a positive correlation between SEPCs contents and HCC tumor diameter in differently differentiated specimens ($P < 0.01$). Hepatocarcinogenesis was determined by an unbalanced angiogenesis process with an augmented production of proangiogenesis factors (drivers of vessel growth and maturation) by tumor cells and adjacent cells, including VEGF, platelet-derived growth factor (PDGF), *et. al.*³⁴⁻³⁶. Tumor size was closely related with tumor angiogenesis, which provided the nutrition carrying blood for tumor growth. In this study, the localization of SEPCs was proved within blood vessels' abundant areas in tumor nodules, and the positive correlation between SEPCs expression and HCC tumor diameter was also certified. The above findings indicated that SEPCs were related to tumor angiogenesis. In fact, most currently approved treatments for advanced HCC in the first- and second-line settings target angiogenic pathways. Of the known or potential angiogenic pathways in tumors, the VEGF/VEGF receptor (VEGFR) signaling pathway has been validated as a drug target in HCC³⁷. Therefore, SEPCs might serve as a potential antiangiogenic therapeutic target of HCC.

Furthermore, results from this study revealed that the SEPCs in low differentiation HCC patients were significantly more than those in both moderate and high differentiation HCC patients ($P < 0.01$), which indicated SEPCs could be a risk factor for prognosis and metastasis. This is also the first study demonstrating that SEPCs is related to differentiation of HCC. Differentiation was closely related with tumor invasion and metastasis in virous tumors including HCC. Thus, SEPCs could be used as a potential prognostic marker by distinguishing poorly differentiated from well differentiated HCC.

Though that SEPCs were in close connection with HCC progression, the sample volume and the follow-up data were limited, and more samples and follow-up data were required to validate our conclusion in further studies. In addition, the underlying mechanisms regarding the roles of SEPCs in the tumor angiogenesis, and progression of HCC remained to be established.

Conclusions

Considering that the contents of SEPCs were associated with tumor marker levels, tumor sizes, differentiation grades of HCC, SEPCs might be applied as a marker for HCC diagnosis, a risk stratification index to predict the prognosis and metastasis of HCC and therapeutic target for HCC.

Abbreviations

AFP, α -fetoprotein; BM SEPCs, bone marrow (BM)-derived sinusoidal endothelial cell progenitor cells; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; LSECs, liver sinusoidal endothelial cells; SEPCs, sinusoidal endothelial cell progenitor cells; VEGF, vascular endothelial growth factor.

Declarations

- Ethics approval and consent to participate

This study was performed in accordance with clinical study protocols and the principles of the Declaration of Helsinki (modified 2000) and approved by the Research Care and Ethics Committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (No. SPPHCT2017–MED-10). Written informed consent was obtained from all patients or their relatives.

- Consent for publication

Consent for publication was obtained from the person whose blood and tissue samples were reported in the manuscript.

- Availability of data and material

The immunohistochemical results of the primary HCC were obtained from the pathology department, where the samples were conserved, immunofluorescence results were kept in the department of basic medical science, and the blood indexes were obtained from the clinical data management system in Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital.

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- Authors' contributions

Ya-xing Feng, Wei Li, Xu-dong Wen, Ning Zhang, Wei-hui Liu, and Zhan-yu Yang designed the study; Ya-xing Feng, Wei Li, Xu-dong Wen, and Ning Zhang contributed to acquisition of data; Ya-xing Feng and Wei Li performed the experimental research. Xu-dong Wen, Ning Zhang, Ya-xing Feng, and Wei Li were in charge of data statistical analysis; Zhan-yu Yang, Ning Zhang, and Wei-hui Liu, Ya-xing Feng, and Xu-dong Wen performed the surgical procedures; Zhan-yu Yang and Wei-hui Liu took charge of critical revision of the manuscript and language polishing; Ya-xing Feng, Wei Li, Xu-dong Wen, Ning Zhang jointly drafted the manuscript; Zhan-yu Yang and Wei-hui Liu did administrative and technical supervision.

- Acknowledgements

None.

- Conflicts of interest

The authors declare no competing interests.

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Tables

Table 1. Demographics and clinic-pathological characteristics. of HCC patients.

Variable	Classification	Number
Age (year)	≥50	25
	≤50	20
Gender	Male	41
	Female	4
Hepatitis B infection	Yes	39
	No	6
Differentiation grade	High	9
	Moderate	9
	Poor	27
Metastasis	Yes	34
	No	11

Table 2. Liver function indexes in patients with hepatocellular carcinoma.

Variable	Classification	Number
Total Protein (g/L)	Normal (65-85)	27
	Low (≤ 65)	18
Albumin (g/L)	Normal (40-55)	31
	Low (≤ 40)	14
Total bilirubin ($\mu\text{mol/L}$)	Normal (5-28)	38
	High (≥ 28)	7
Direct bilirubin ($\mu\text{mol/L}$)	Normal (0-7)	26
	High (≥ 7)	19
Indirect bilirubin ($\mu\text{mol/L}$)	Normal (3-23)	39
	High (≥ 23)	6
ALT (U/L)	Normal (9-60)	30
	High (≥ 60)	15
AST (U/L)	Normal (15-45)	22
	High (≥ 45)	23

Abbreviation: ALT=glutamate-pyruvate transaminase, AST=aspartate aminotransferase.

Table 3. The expression levels of tumor markers in hepatocellular carcinoma patients.

Variable	Classification	Number
AFP (ng/ml)	Normal (0-9)	4
	High (≥ 9)	41
CA-199 (U/ml)	Normal (0-35)	30
	0-35	
	High (≥ 35)	15
	≥ 35	
CEA (ng/ml)	Normal (0-10)	35
	High (≥ 10)	10
CA-125 (U/ml)	Normal (0-35)	33
	High (≥ 35)	12

Abbreviation: AFP= α -fetoprotein, CEA=carcinoembryonic antigen.

Figures

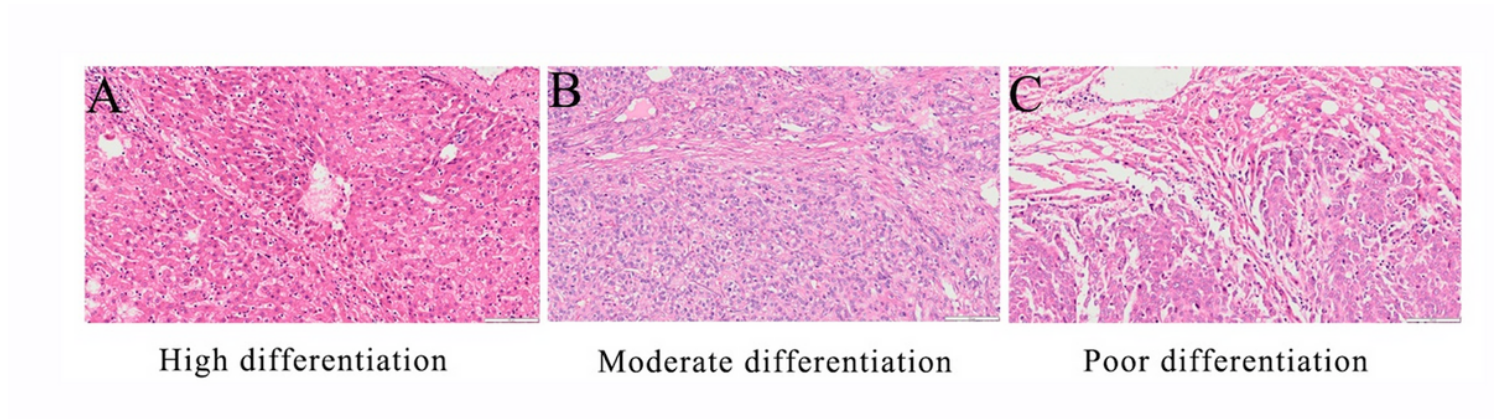


Figure 1

Different degrees of differentiation in hepatocellular carcinoma (HCC). Typical cases of highly differentiated (A), moderately differentiated (B) and poorly differentiated (C) HCC tissues were determined by hematoxylin-eosin staining.

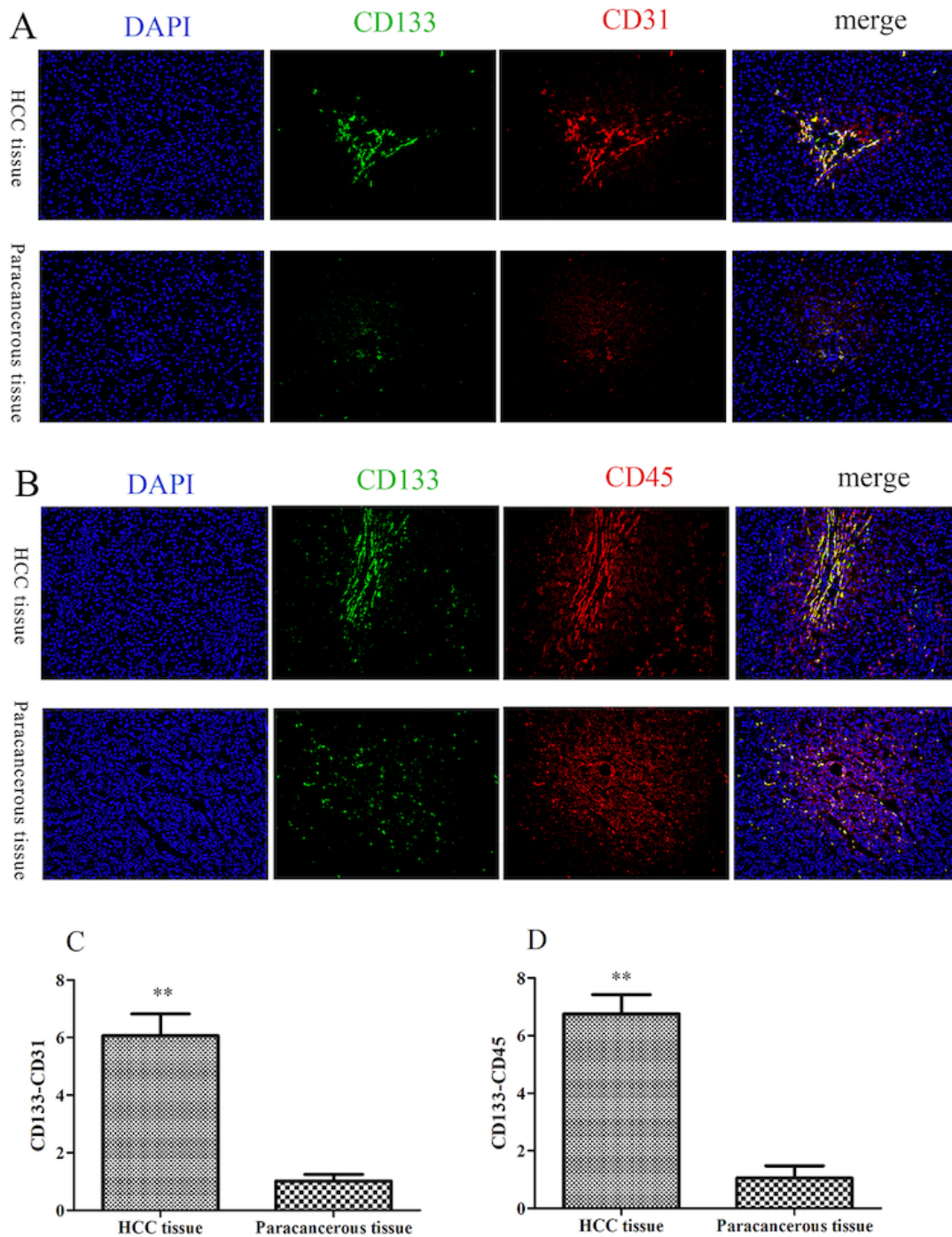


Figure 2

Localization of sinusoidal endothelial cell progenitor cells (SEPCs) in the liver tissues of HCC patients. Representative immunofluorescence images of SEPCs' markers (CD133, CD31, and CD45) in liver tissues from HCC patients were found abundantly around the blood vessels in tumor tissues, and very few SEPCs in corresponding tumor-adjacent tissues (A, B). Magnification: 100×. Markers' expression levels of SEPCs

(CD133, CD31, and CD45) in tumorous are significantly higher than those in adjacent nontumorous liver tissues (C, D). **: P<0.01.

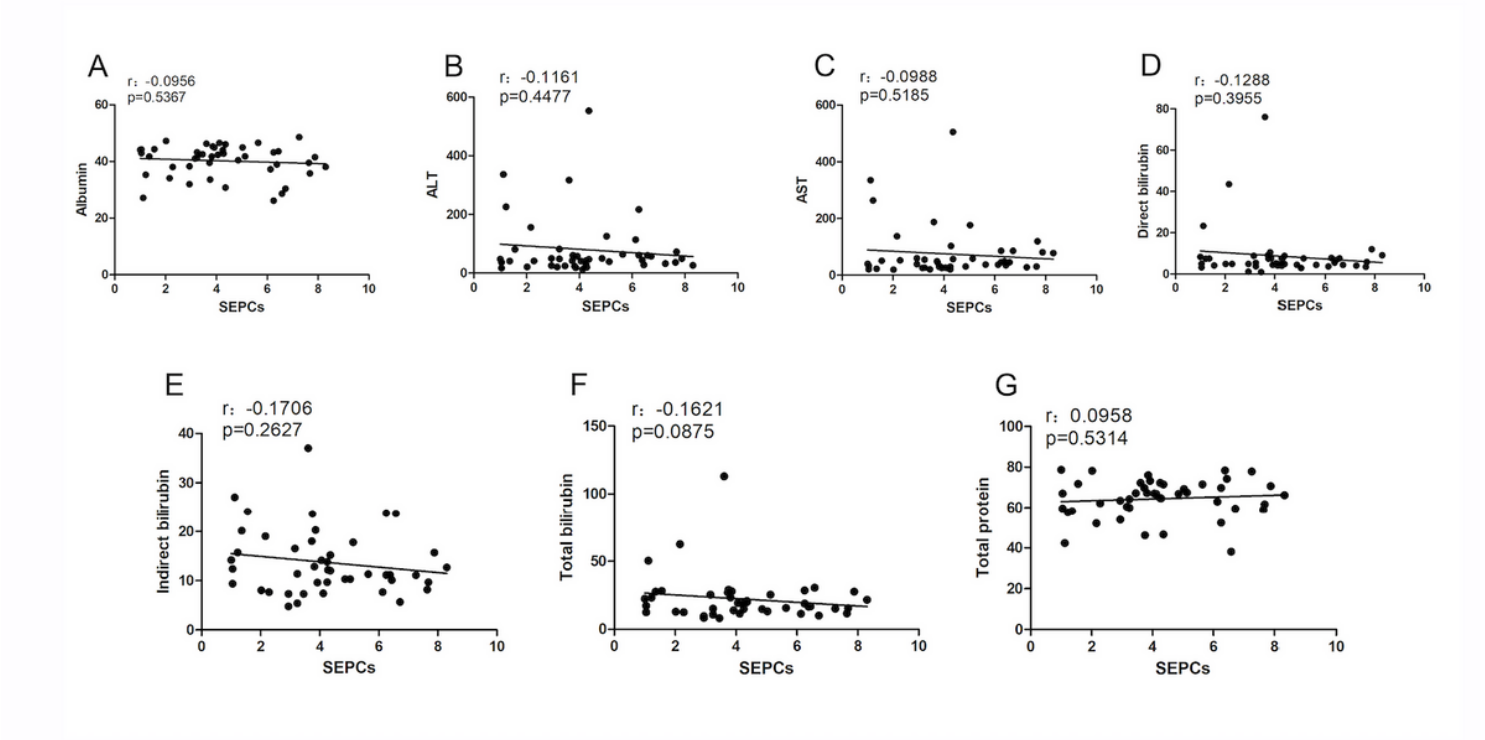


Figure 3

The Spearman correlations between SEPCs and liver functional parameters. There were no significant correlations of SEPCs expressions and albumin (A), alanine aminotransferase (B), glutamic-oxalacetic transaminease (C), direct bilirubin (D), indirect bilirubin (E), total protein (F), and total protein (G) within the 45 hepatocellular carcinoma patients.

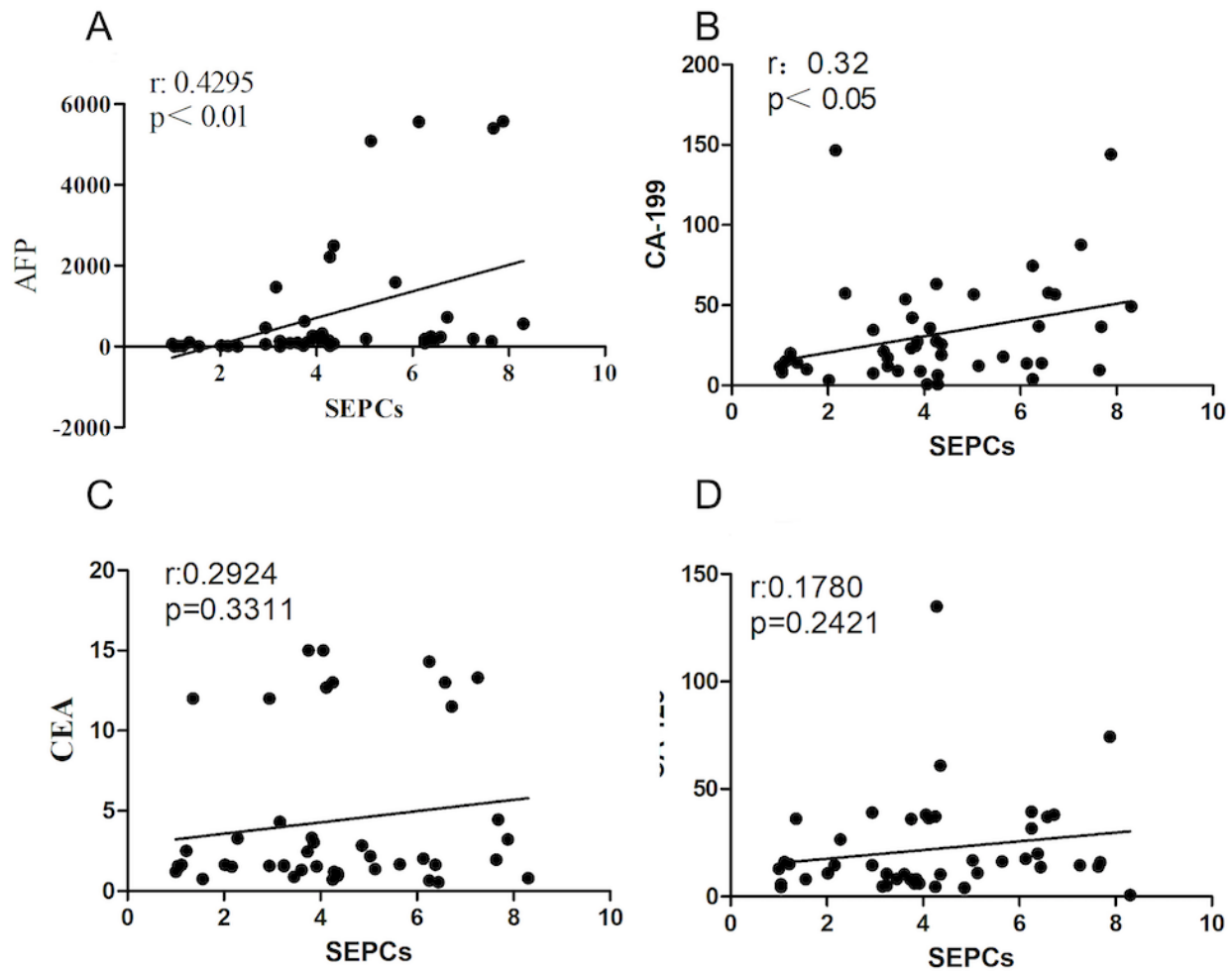


Figure 4

The spearman correlations between SEPCs and tumor-related markers. There were positive correlations between SEPCs expression and alpha-fetoprotein (A) and CA199 (B) level, but no correlation between SEPCs expression and carcinoembryonic antigen (C), CA125 level (D).

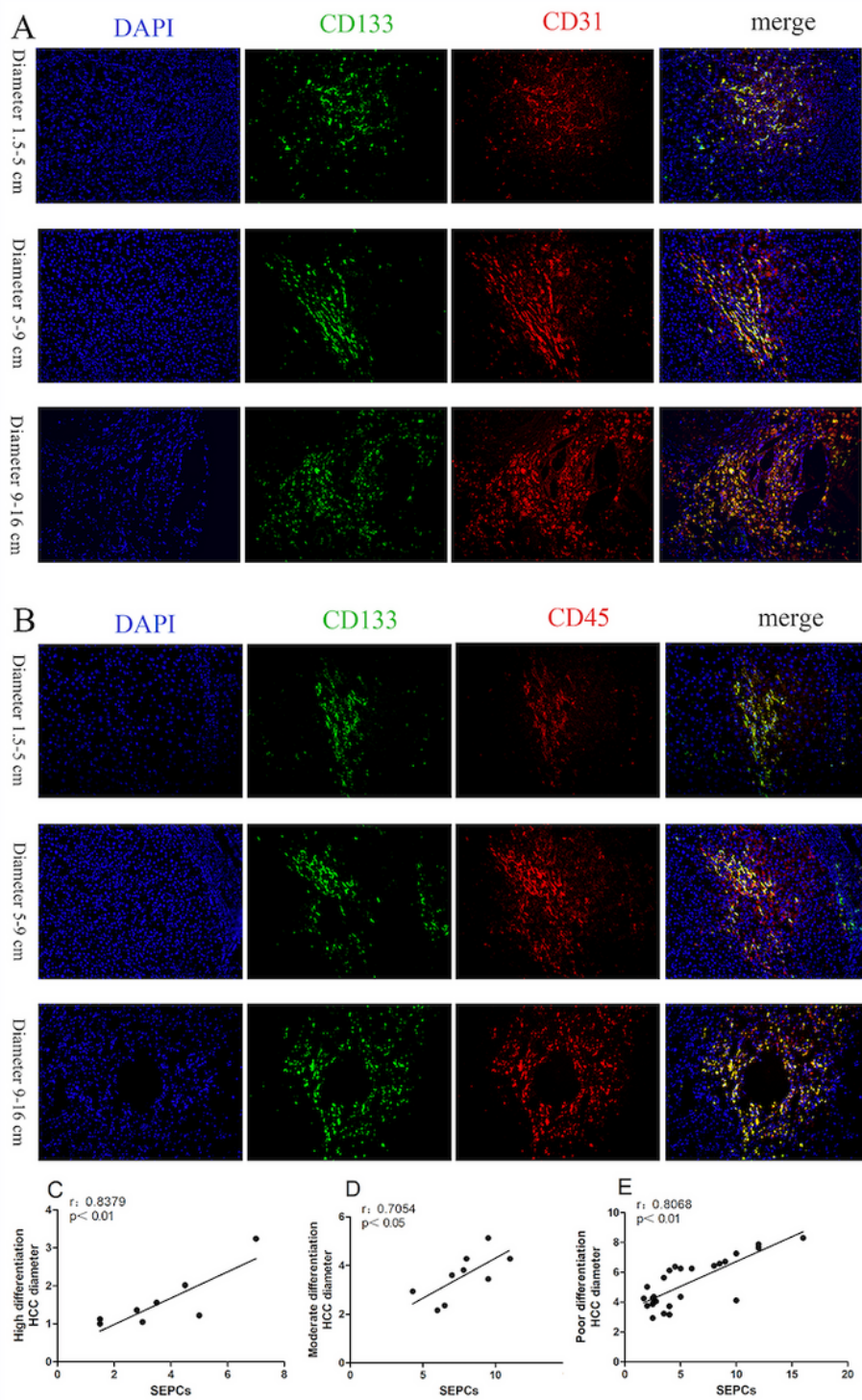


Figure 5

SEPCs expressions in HCC lesions with different sizes and their correlations with sizes in different differentiations. Representative immunofluorescence images of SEPCs markers (CD133, CD31, and CD45) in differently sized HCC lesions were displayed (A, B). Magnification: 100×. Significant correlations between SEPCs expression and tumor sizes in highly differentiated (C), moderately differentiated (D), and poorly differentiated (E) specimens were achieved.

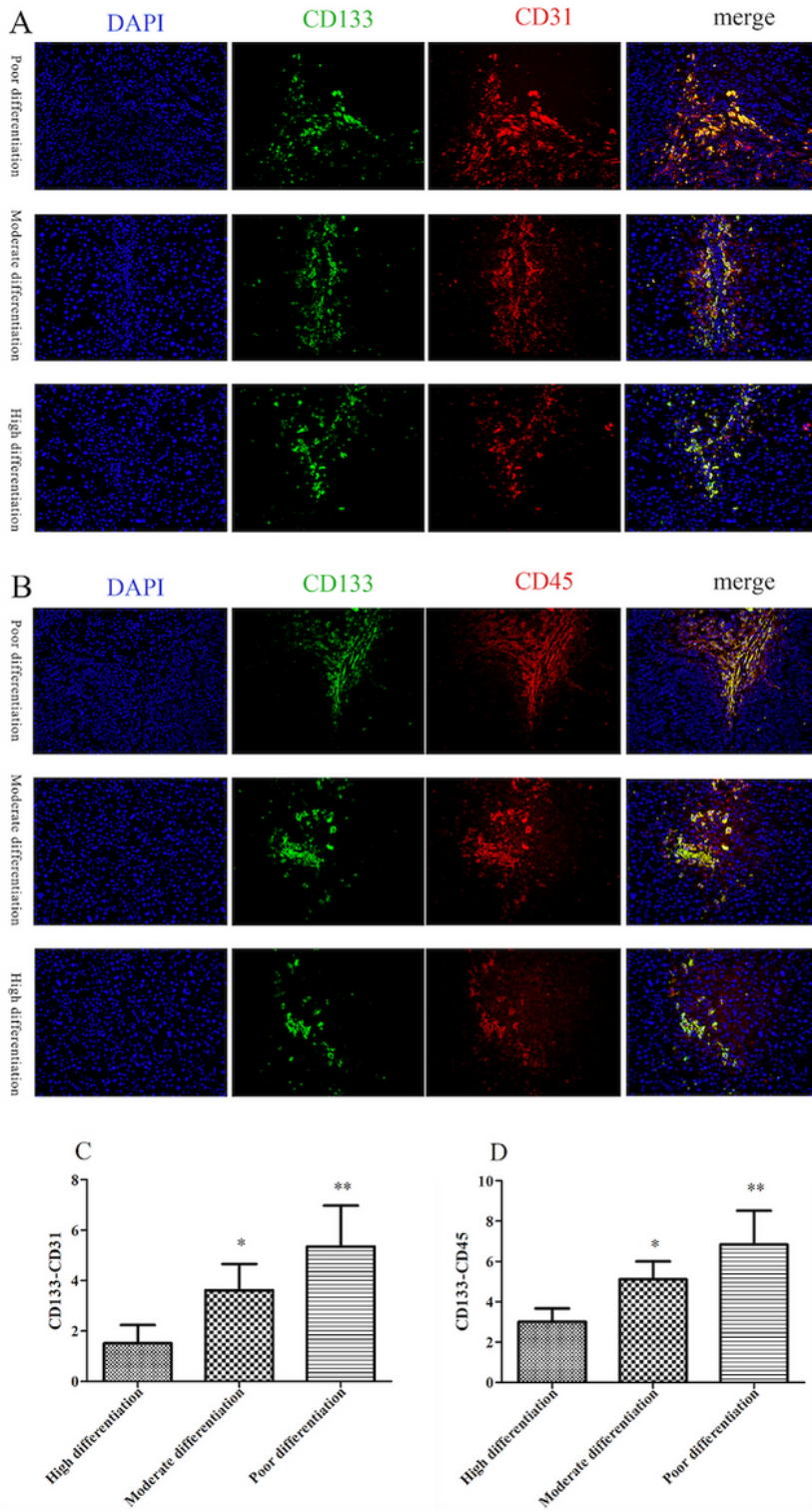


Figure 6

SEPCs expressions in HCC lesions with different differentiations and their correlations. Representative immunofluorescence images of SEPCs markers (CD133, CD31, and CD45) in different differentiations of HCC specimens (A, B). Magnification: 100×. Quantities of SEPCs in HCC specimens with different differentiations were analyzed. Significant differences of SEPCs expressions were found among high,

moderate, and poor differentiation groups in the CD133-CD31 group (C), as well as the CD133-CD45 group (D). *: $P < 0.05$, **: $P < 0.01$.