Identification and Clinical Significance of Mobilized Endothelial Progenitor Cells in Tumor Vasculogenesis of Hepatocellular Carcinoma

Decai Yu,[1,2] Xitai Sun,[1,2] Yudong Qiu,[1,2] Jianxing Zhou,[2] Yafu Wu,[2] Lingyuan Zhuang,[1] Jun Chen,[1] and Yitao Ding[1,2]

Abstract

Purpose: To investigate the distribution, frequency, and clinical significance of mobilized endothelial progenitor cells (EPC) in hepatocellular carcinoma (HCC).

Experimental Design: In healthy controls and patients with HCC, the frequency of circulating EPCs was determined by colony-forming assays, fluorescence-activated cell sorting, and real-time PCR. One hundred sixty-five – amino acid form of vascular endothelial growth factor and platelet-derived growth factor-BB in plasma and tissue were quantified by ELISA. The distribution and frequency of EPCs were evaluated by immunofluorescence, immunohistochemistry, and real-time PCR in normal liver (n = 8), and tumor tissue (TT), adjacent nonmalignant liver tissue (AT), and tumor-free tissue 5 cm from the tumor edge (TF) from 64 patients with HCC. Clinicopathologic data for these patients were evaluated.

Results: Compared with values for healthy controls, colony-forming unit scores were higher in the peripheral blood of patients with HCC. Plasma 165-amino acid form of vascular endothelial growth factor and platelet-derived growth factor-BB correlated with the expression level of the *AC133* gene, which was also higher in the peripheral blood of patients with HCC. Immunohistochemical analysis showed that EPCs were incorporated into the microvessels in cirrhotic and tumor tissue. Compared with normal liver (9.00), increased $AC133^+$ microvessel density (microvessels/0.74 mm^2) was found in TT (53.56), AT (84.76), and TF (48.33). The levels of *AC133* gene expression and AC133-microvessel density in AT, which were the highest among four groups, correlated with clinicopathologic variables (the absence of tumor capsule, venous invasion, proliferating cell nuclear antigen intensity, and early recurrence).

Conclusions: Mobilized EPCs participate in tumor vasculogenesis of HCC. *AC133* gene or antigen in peripheral blood and liver tissue could be used as a biomarker for predicting the progression of HCC.

Angiogenesis, the formation of new capillaries from preexisting vasculature, is essential for tumor growth and metastasis and represents an important prognostic indicator in hepatocellular carcinoma (HCC; ref. 1). Recent evidence suggests that endothelial cells from neighboring preexisting capillaries are not the only source of increased tumor vascularization. Bone marrow–derived endothelial progenitor cells (EPC) are also thought to contribute to the formation of new vessels in tumors, a process known as vasculogenesis (2). EPCs resemble embryonic angioblasts, which characteristically migrate, proliferate, and differentiate into mature endothelial cells (3). In general, EPCs can be identified as cells that simultaneously express the cell surface markers CD34, AC133/CD133, and kinase insert domain-containing receptor (4, 5).

Arbab et al. (6) and Shirakawa et al. (7) used mouse tumor models to show that bone marrow–derived EPCs are involved in tumor vasculogenesis and tumor growth, especially in early phases. In clinical investigations, two studies have reported that EPCs are recruited and homed with high specificity to solid tumors (8, 9). Reports on the numerical contribution of EPCs to vessel growth are variable, ranging from low (<0.1%) to high (up to 50%), likely dependent on the type of angiogenesis model used (10, 11). Moreover, unselected bone marrow cells (12) and endothelial progenitor-like cells (13) were engineered as vectors to hinder tumor angiogenesis and slow the growth of tumors. Because these conclusions support the hypothesis that EPCs play a functional role in vasculogenesis and growth of human solid tumors, there are possibilities that EPCs can be used as diagnostic or prognostic markers and as vectors for targeting cancers (11, 14).

HCC is a highly vascularized tumor. The majority of HCC tissue samples exhibit strong expression of proangiogenic

Authors' Affiliations: [1]Institute of Hepatobiliary Surgery and [2]Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital, School of Medicine, Nanjing University, Nanjing, Jiangsu Province, P. R. China

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Requests for reprints: Yitao Ding, Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital, School of Medicine, Nanjing University, Nanjing, Jiangsu Province, P.R. China. Phone: 86-25-83304616, ext. 66866; Fax: 86-25-83317016; E-mail: drdingyitao@sina.com.cn.

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factors, such as the 165-amino acid form of vascular endothelial growth factor ($VEGF_{165}$), platelet-derived growth factor-BB (PDGF-BB), insulin-like growth factor II, and basic fibroblast growth factor (15–18). These proangiogenic factors are important in the neovascularization, growth, and development of human HCC. These factors are also involved in activating, mobilizing, and recruiting EPCs from the bone marrow (19) and in promoting differentiation of EPCs into endothelial cells in some ischemic and tumor diseases (20, 21).

Recently, Poon et al. (22) reported that the level of circulating EPCs was elevated in patients with HCC and might correlate with the aggressiveness of the tumor. This was shown by a short-term culture assay involving scoring of colony-forming units (CFUs) of EPCs in the peripheral blood. Furthermore, there were positive correlations between the number of circulating EPCs and serum α-fetoprotein, VEGF, and interleukin-8 levels. However, there is no report providing evidence that EPCs participate in the neovascularization of HCC. Of note, animal models and clinical investigations have revealed that bone marrow–derived cells participate in neovascularization by committing to sinus endothelial cells in liver regeneration resulting from exposure to CCl_4 or from partial hepatectomy (23, 24).

Therefore, we hypothesized that EPCs were mobilized from the bone marrow of HCC patients by proangiogenic factors ($VEGF_{165}$ and PDGF-BB) and sustained the increase of vasculogenesis in HCC. We examined the number of EPCs in the peripheral blood and the distribution of EPCs in the liver of patients with HCC. We also analyzed the relationship between the level of *AC133* gene expression in liver and various clinicopathologic variables.

Materials and Methods

Patients and samples

Between January 2004 and August 2006, 64 patients were enrolled in the study in the Department of Hepatobiliary Surgery of Drum Tower Hospital. None of the patients had received preoperative treatment. Preoperative clinical and laboratory data, including routine liver biochemistry, a complete blood count, hepatitis B virus infection, and serum α-fetoprotein level, were prospectively assembled for each patient in a computerized database. Before surgery, peripheral blood was collected in EDTA-containing tubes through 21-gauge needles for colony-forming assays, fluorescence-activated cell sorting, plasma preparation, and RNA isolation. The peripheral blood from 20 healthy male volunteers (median age, 45 years; range, 38-59 years) and normal liver tissues from 4 liver transplant donors and 4 patients without liver disease served as healthy controls (HC). The research ethics committee of our hospital approved the protocol and informed consent was obtained from all participants.

Proangiogenic factors, such as VEGF (25) and hepatic growth factor (26), have higher expression in the surrounding liver than in the tumor itself, which are involved in mobilizing and recruiting EPCs (20, 21). Therefore, three different types of tissues from each HCC patient were assembled immediately after surgical resection: tumor-free tissue >5 cm far from the tumor edge (TF), adjacent nonmalignant tissue within 2 cm (AT), and tissue from the tumor (TT). Areas of tissue necrosis and hemorrhage were excluded. All of the tissue samples were snap frozen immediately after resection and kept in liquid nitrogen until they were used for experiments. Histopathologic examination of all specimens was done by a senior pathologist with experience in HCC pathology (Prof. L.H. Zhang), who was unaware of the preoperative clinical data and immunostaining results. Tumors were graded according to the criteria described by Edmonson and Steiner (27). Serial sections of the tumors and surrounding liver were examined to identify any tumor encapsulation, microscopic venous invasion, and microsatellite lesions. The degree of HCC invasiveness was verified according to the invasiveness scoring system for HCC (28).

EPC colony-forming assay

Peripheral blood mononuclear cells from HC and patients with HCC were suspended in EGM-2 MV BulletKit (CC-3202, Clonetics/BioWhittaker). After 48 h, nonadherent cells were collected and plated onto six-well plates coated with fibronectin (Sigma). The culture medium was changed every 3 days. On the 7th day, EPCs were identified with an inverted fluorescent microscope (Carl Zeiss, Inc.) by the uptake of 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (Molecular Probes) and the binding of FITC-labeled Ulex europaeus agglutinin I (Sigma; ref. 29). CFUs, defined in the manner of Hill et al. (30) as consisting of multiple thin, flat cells emerging from a cluster of round cells, were quantified on the 7th day in a minimum of six fields (original magnification, ×40) per well by two observers without knowledge of the subjects' clinical profiles.

Fluorescence-activated cell sorting analysis

Circulating EPCs were measured by fluorescence-activated cell sorting analysis in unselected peripheral blood cells from HC and patients with HCC. EDTA-anticoagulated blood (100 μL) was incubated for 30 min at 4°C with 5 μL phycoerythrin-conjugated anti-AC133 (Miltenyi Biotec), PerCP-conjugated anti-CD45, and FITC-conjugated anti-CD34 (B&D). IgG1-FITC and IgG2α-phycoerythrin antibodies (B&D) served as isotype controls for each procedure. After incubation, cells were lysed and washed with PBS before analysis. For each sample, a minimum of 50,000 events was acquired. Circulating $CD34^+$ and $CD133^+$ mononuclear cells were tentatively classified as EPCs. Quantitative fluorescence analysis was done using FACSCalibur and WinMDI software (B&D).

Immunofluorescent staining of tissue sections

Frozen liver tissue sections were cut to a thickness of 4 or 20 μm. Sections were fixed in cold acetone for 5 min, air dried, and immersed in PBS. Sections were incubated with phycoerythrin-conjugated anti-AC133 (1:100) and FITC-conjugated anti-CD34 (1:100) at 37°C for 2 h. PBS was substituted for antibodies as a negative control. Fluorescently labeled cells were detected in the vessel wall via a fluorescence microscope using a Zeiss Axiovert scope (Carl Zeiss) and confocal laser scanning microscopy (MRC 1024, Bio-Rad, Inc.). A series of 1-μm optical sections through the entire thickness of the tissue were obtained using a 40× objective, and Z series were constructed from these sections.

Immunohistochemistry of frozen sections. Consecutive frozen liver tissue sections of 4-μm thickness were cut in a cryostat, fixed, and incubated with AC133 (1:100; Miltenyi Biotec) or CD34 (1:300; Santa Cruz Biotechnology) monoclonal mouse anti-human antibody monoclonal antibodies at 4°C overnight. A subsequent reaction was done with biotin-free horseradish peroxidase enzyme–labeled polymer from an EnVision plus detection system (DAKO). Positive reactions were visualized with diaminobenzidine solution followed by counterstaining with hematoxylin. Negative controls were obtained by substituting the primary antibodies with PBS.

Immunohistochemistry of paraffin-embedded sections. Conventionally processed and embedded sections cut at a thickness of 4 μm were deparaffinized, blocked, and incubated at 4°C overnight with anti–proliferating cell nuclear antigen (PCNA) antibody (DAKO). A subsequent reaction was done as described above.

Determination of $CD34^+$ and $AC133^+$ microvessel density. Microvessel density (MVD) was evaluated according to Gasparini's criteria (31) on an Olympus microscope (C-X31) with an Olympus camera (C-5050Z) by two independent observers who were blinded to the patients' clinicopathologic data. Any brown-stained $CD34^+$ cells or

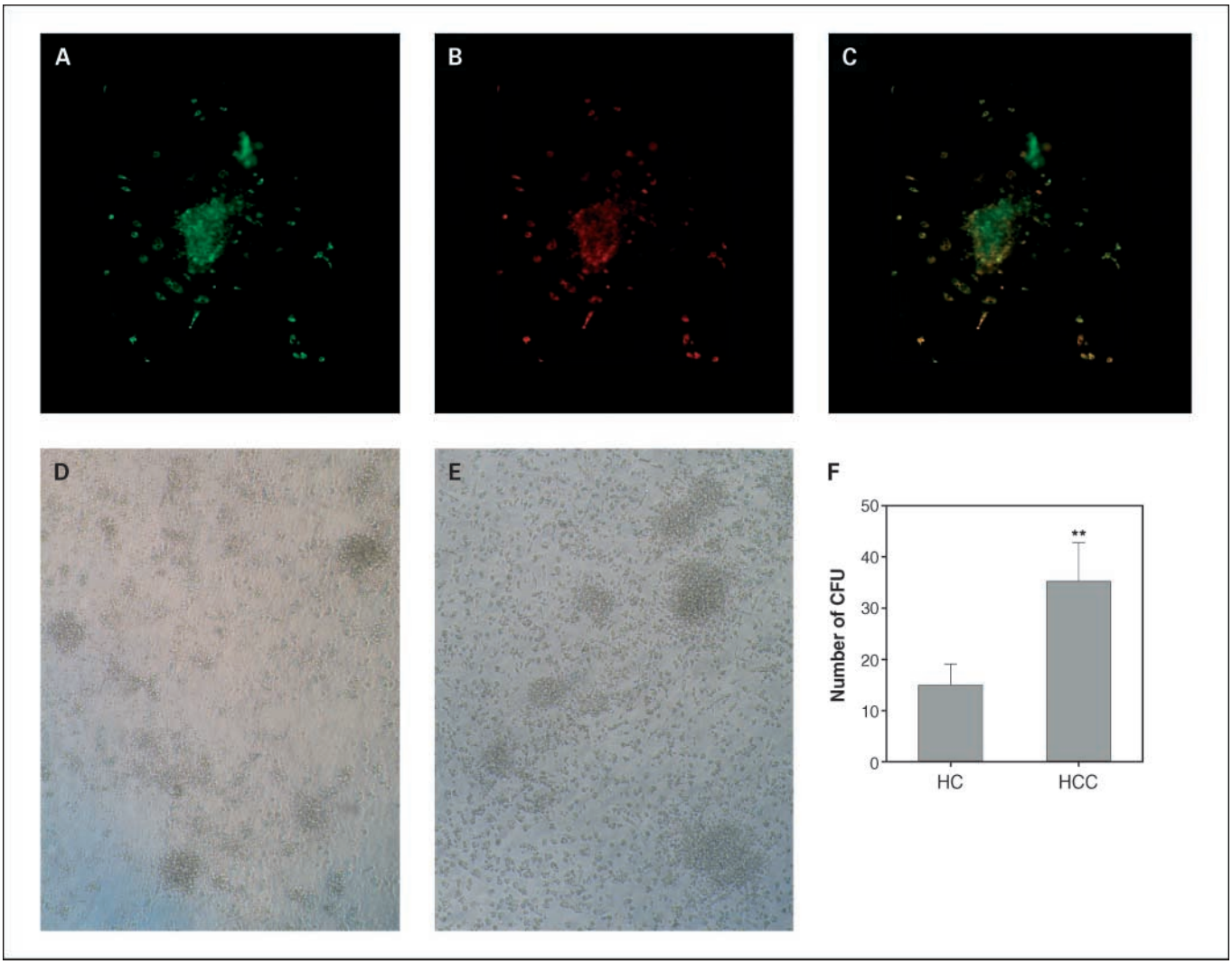

Fig. 1. Identification of EPCs *in vitro* and comparative analysis. Peripheral blood mononuclear cells were cultured and identified under fluorescent microscope on the 7th day for Ulea-1 binding (*A, green*) and 1,1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein uptake (*B, red*). Double-positive cells, appearing in yellow in the overlay (*C*), were identified as differentiating EPCs. Representative CFUs from a HC (*D*) and a patient with HCC (*E*) were shown on the 7th day of the culture. Magnification, ×40. *F,* number of CFUs per 1 $\times 10^6$ peripheral blood mononuclear cells in HC ($n = 5$) and patients with HCC ($n = 5$). Columns, number of CFUs; bars, SD. **, $P < 0.01$.

$AC133^+$ cells that were separated from adjacent microvessels, tumor cells, and connective elements were counted as one microvessel, irrespective of the presence of a vessel lumen. The mean microvessel count of the five most vascular areas was taken as the MVD, which was expressed as the absolute number of microvessels per 0.74 mm^2 (×200 field). $PCNA^+$ cells were counted from representative areas of the sections and expressed as a percentage. The intensity of immunostaining was categorized as follows: -, negative; +, low; and ++, moderate-high.

Real-time PCR

RNA isolation and reverse transcription. Acid guanidine thiocyanate-phenol-chloroform extraction was used to isolate total RNA from liver tissues. Total RNA of peripheral blood was extracted from peripheral blood mononuclear cells by Trizol reagent (Life Technologies) according to the manufacturer's instructions. With random hexamer primers, the maximum allowed volumes of RNA samples were transcribed with ExScript RT reagent kit (TaKaRa) according to the manufacturer's protocol. RNA samples without reverse transcription were used as negative controls.

PCR. Primers and probes for human *AC133*, *CD34*, and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) genes were designed with Primer Express 2.0 software (Applied Biosystems) and synthesized by Genecore. The basic information on primers and probes, including gene name, National Center for Biotechnology Information reference, forward primer, reverse primer, probe and its location between two exons, and product size (bp), respectively, are the following: *AC133*, NM_006017, GACCGACTGAGACCCAACATC, AGGTGCTGTTCATGTTCTCCAA, FAM-CAACAGCGATCAAGG-MGB, 7 and 8, and 103; *CD34*, NM_001025109, CCTTGCAACATCTCCCACTAAAC, TTCACTTCTCTGATGCCTGAACA, FAM-ACATCAAGGCAGAAAT-MGB, 3 and 4, and 96; and *GAPDH*, NM_002046, GGGCTGCTTTTAACTCTGGTAAAG, CCATGGGTGGAATCATATTGG, FAM-CCTCAACTACATGGTTTAC-MGB, 1 and 2, and 103. For the amplification of *AC133*, *CD34*, and *GAPDH* genes, real-time PCR was done in triplicate for each sample in a 20 μL reaction mixture, which consisted of template DNA (2 μL), primers (900 nmol/L), probe (250 nmol/L), Mg^{2+} (5 mmol/L), and Ex Taq HS (0.1 units/μL; ExScript Real-time PCR Kit, TaKaRa). PCR was done in a Stratagene Mx3005P instrument using the following thermal cycles: one cycle of 10 s at 95°C, 55 cycles of

5 s at 95°C, and 20 s at 60°C. Amplification efficiency of each individual sample was calculated by version 7.0 of LinRegPCR program (a gift from C.R. Ramakers,[3] Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands). According to the method tested by Pfaffl (32), the relative expression ratio of a targeted gene was calculated based on efficiency and the C_t compared with a reference gene (*GAPDH*).

Measurement of plasma and tumor cytosolic $VEGF_{165}$ and PDGF-BB protein concentration

The isolation of plasma and tumor cytosolic proteins was done as described by Poon et al. (15). Before surgery, peripheral venous blood samples were taken from the patients and centrifuged at 3,000 rpm for 10 min and then stored at -80°C. Protein cytosolic fractions were obtained by homogenization of tissues. Homogenates were lysed with equal volumes of radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.02% NaN_3, 1% Triton X-100, 1% SDS] with Cocktail protease inhibitor (1:200; Sigma) on ice for 30 min and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatants were assayed for cytosolic $VEGF_{165}$, PDGF-BB, and total protein concentration.

$VEGF_{165}$ and PDGF-BB in plasma and liver tissue were quantified by ELISA ($VEGF_{165}$ and PDGF-BB Immunoassay, Lifekey Corp.). Each measurement was made in duplicate, and the $VEGF_{165}$ and PDGF-BB levels were determined from a standard curve generated for each set of samples assayed. The total protein concentration in tissues was determined with the Coomassie plus protein assay reagent (Pierce Chemical Corp.). To correct for variation in total protein concentration, the relative concentrations of $VEGF_{165}$ and PDGF-BB in tissues were calculated by dividing $VEGF_{165}$ and PDGF-BB concentrations by the total protein concentration in the tissue.

Clinicopathologic database and follow-up

All clinicopathologic data were assembled prospectively in a computerized database, and all patients were followed and monitored regularly for tumor recurrence by α-fetoprotein level (monthly) and chest X-ray, together with B ultrasonic or computed tomography scan (every 3 months). The median follow-up time of all patients was 14 months (range, 3-34 months). A diagnosis of recurrence was based on typical imaging appearance in computed tomography scan and an elevated α-fetoprotein level and, if necessary, fine-needle aspiration cytology. All of the patients were followed until death or until the study closing date of October 1, 2006.

Statistical analysis

Data were expressed as mean ± SD with the range given in parentheses. Statistical comparisons were done using the *t* test, ANOVA, and linear regression when data were normally distributed. The Pearson χ^2 test was used to compare the results of two or more subgroups. All statistical procedures were done using SPSS (version 11.5; SPSS, Inc.). Values of $P < 0.05$ were considered statistically significant.

Results

Patient data. In 64 patients (53 males and 11 females; median age, 51 years) who underwent curative resection (57 cases for regular hepatectomy and 7 for orthotopic liver transplantation), the average tumor size was 6.65 ± 4.17 cm (range, 0.8-20 cm). Liver cirrhosis was detected in 60 patients; the remaining 4 patients had chronic hepatitis. The etiologies of underlying liver diseases were hepatitis B in 56 patients, hepatitis C in 1 patient, mixed viral infection in 1 patient, alcoholic cirrhosis in 4 patients, and cirrhosis of unidentified etiology in 3 patients. According to International Union Against Cancer recommendations (2002; ref. 33), 25 patients were classified as stage I, 12 patients as stage II, 26 patients as stage III, and 1 patient as stage IV. Fifty-two patients were in child's class A, 11 in class B, and 1 in class C.

Mobilized EPCs are increased in the peripheral blood of patients with HCC compared with HC. To date, no clear definition of EPCs exists and their extremely low number makes isolation difficult. Therefore, based on recent investigations (34), we determined the number of circulating EPCs by a functional assay (colony formation, 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein uptake and for Ulea-1 binding) and quantitative analysis of phenotypes (fluorescence-activated cell sorting and real-time PCR).

First, circulating EPCs were evaluated in the peripheral blood of patients with HCC and HC by colony-forming assay. Representative CFUs were observed and counted on the 7th

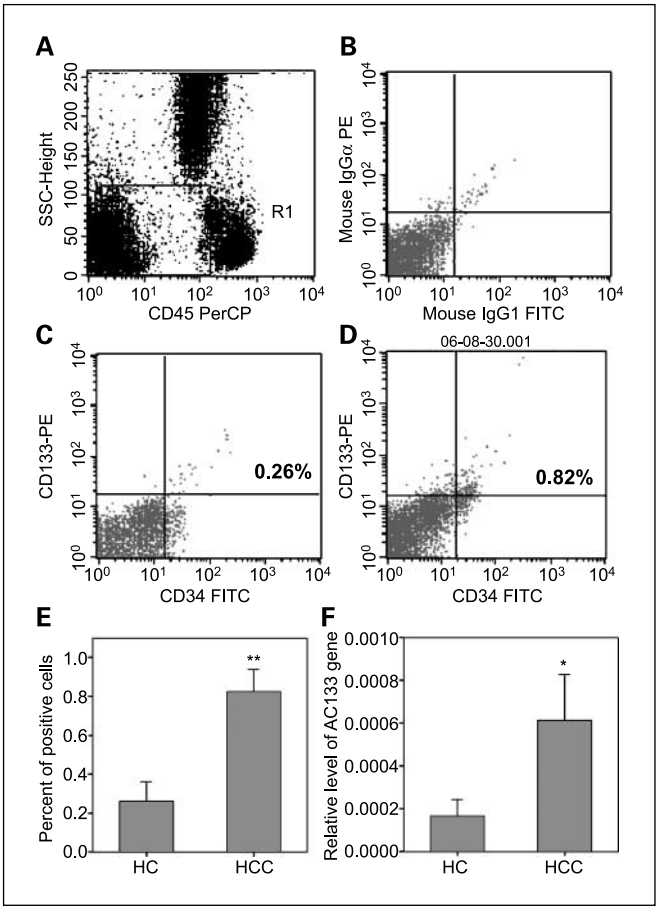

Fig. 2. Frequency of $AC133^+$ and $CD34^+$ cells in patients with HCC compared with HC. *A,* forward and side scatter evaluation with lack of the hematopoietic marker CD45 was used to generate the analysis gate. *B,* negative controls stained with phycoerythrin- or FITC-labeled IgG isotype controls. The percentage of the population expressing the indicated double antigens ($AC133^+$ and $CD34^+$) from HC (*C*) and patients wit HCC (*D*) was shown in each quadrant. *E,* the percentage of $CD34^+$ and $AC133^+$ cells in HC ($n = 6$) and patients with HCC ($n = 11$). *F,* the relative level of *AC133* gene in peripheral blood of HC ($n = 5$) and patients with HCC ($n = 12$). Columns, percentage of positive cells (*E*) and relative level of *AC133* gene (*F*); bars, SD. *, $P < 0.05$; **, $P < 0.01$.

[3] c.r.ramakers@amc.uva.nl

day. First, EPCs were identified as adherent cells that were positive for both 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein uptake and Ulea-1 binding as determined by fluorescent microscopy (Fig. 1A-C). Representative EPC colonies were characterized by a central cluster of round cells surrounded by radiating, thin, flat, elongated cells (Fig. 1D, from a HC; Fig. 1E, from a patient with HCC). Relative to HC (15.00 ± 4.06, $n = 5$), mean CFU scores from HCC patients (35.20 ± 7.56, $n = 5$) were higher on the 7th day ($P = 0.001$; Fig. 1F).

Moreover, the mean percentage of $CD34^+$ and $AC133^+$ cells in the peripheral blood of patients with HCC (0.82% ± 0.12, $n = 11$) was elevated relative to HC (0.26% ± 0.10, $n = 6$; $P = 0.002$; Fig. 2A-E). In addition, the relative level of *AC133* gene expression in the peripheral blood of patients with HCC (0.00061 ± 0.00021, $n = 12$) was higher than in HC (0.00017 ± 0.00008, $n = 5$; $P = 0.039$; Fig. 2F). In conclusion, there are more mobilized EPCs in the peripheral blood of patients with HCC than in HC.

$AC133^+$ and $CD34^+$ cells incorporate into vessels in tumor tissue. In frozen sections from 10 different tumor specimens, cells expressing AC133 and CD34 antigens were simultaneously identified in endothelium by direct immunofluorescence staining using anti-AC133 and CD34 antibodies. In tumor tissue, all of the $AC133^+$ cells were positive for the CD34 antigen in portal veins and microvessels. The double-positive cells were incorporated into vessel walls of different sizes, such as portal veins (Fig. 3A and B) and microvessels (Fig. 3B and C) in tumors. Additionally, a projection made from segmented confocal image data indicated that $AC133^+$ and $CD34^+$ cells incorporated into portal veins and microvessels in tumor tissue (Fig. 3D-I).

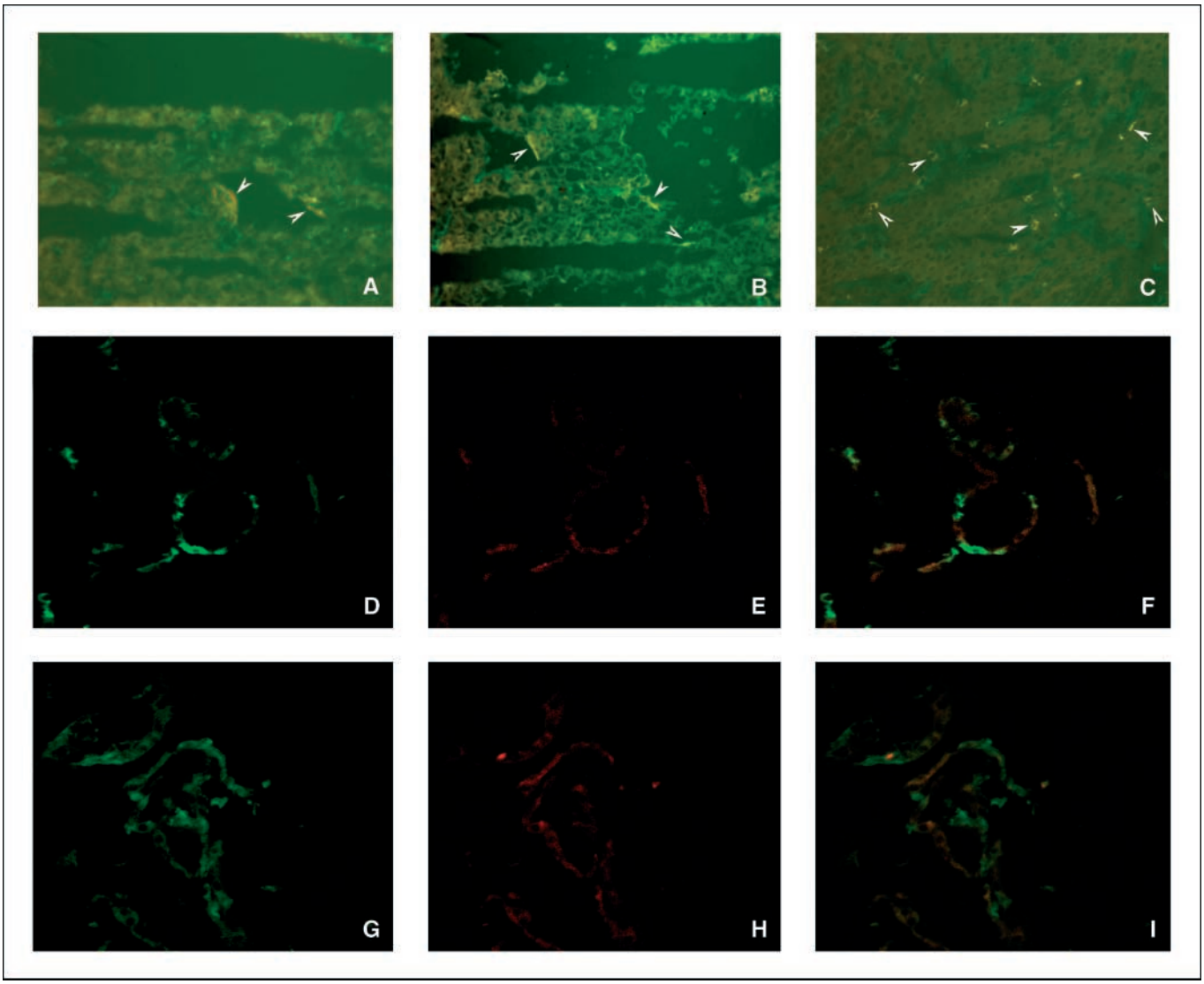

Fig. 3. Location of $AC133^+$ and $CD34^+$ cells in liver cancer. Frozen sections of tumor specimens labeled with FITC-conjugated anti-CD34 (*green*) and phycoerythrin-conjugated anti-AC133 (*red*). *A* to *C,* representative merged images showed that all of $AC133^+$ cells are localized $CD34^+$ endothelium, such as portal veins (*A* and *B*) and microvessels (*B* and *C*) in tumor tissue. Arrows, double-positive cells (*yellow*). Two confocal Z series were shown (*D-F*, portal vein; *G-I*, microvessels). *D* and *G,* FITC-labeled CD34 binding (*green*) specifically to the tumor vasculature; *E* and *H,* phycoerythrin-AC133 antibody binding (*red*) within those same cells; *F* and *I,* merged image of the two previous images, showing colocalization of CD34 and AC133 (*yellow*) to the vessels. Magnification, ×200.

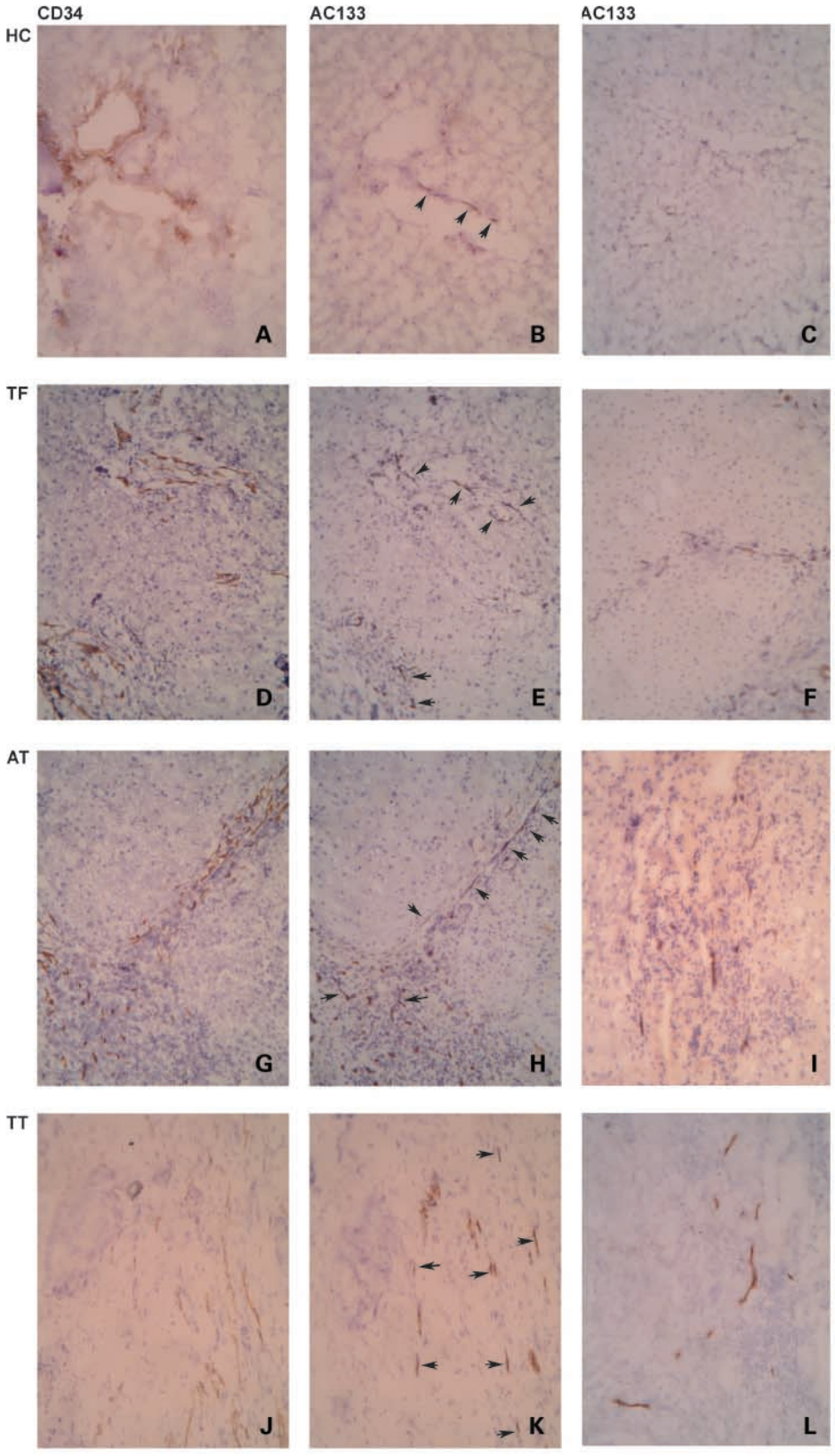

Fig. 4. Distribution of AC133 and CD34 antigen in HC, TT, AT, and TF. Representative immunostaining images were presented for CD34 (*A, D, G*, and *J*) and AC133 (*B, E, H*, and *K*) in two consecutive 4-μm sections of HC (*A* and *B*), TF (*D* and *E*), AT (*G* and *H*), and TT (*J* and *H*), respectively. Arrows, double-positive cells. $AC133^{+}$ cells incorporating into microvessels in HC, TF, AT, and TT were presented, respectively (*C, F, I*, and *L*). Magnification, ×200.

EPCs in patients with HCC were recruited and incorporated into the microvessels in TF, AT, and TT and were especially abundant in AT. In samples from HC, consecutive sections stained for the AC133 and CD34 antigens showed that the $AC133^+$ and $CD34^+$ cells were incorporated mostly into the vessels of portal areas (Fig. 4A-C). $AC133^+$ and $CD34^+$ cells were incorporated into vessels of different sizes, most frequently in microvessels in samples of cirrhotic and tumor tissue in TF (Fig. 4D-E), AT (Fig. 4G-H), and TT (Fig. 4J-K). Highlighted microvessels ($AC133^+$) showed two patterns of expression in TF, AT, and TT sections. The first showed branching; in the second, the microvessels were small without apparent lumina (endothelial sprouts; Fig. 4F, I, and L). Of note, there were some $AC133^+$ cells in stroma septa (Fig. 4H). Compared with AC133-MVD in normal liver samples (9.00 ± 0.54, $n = 4$), increased AC133-MVD (microvessels/0.74 mm^2) was found in 18 patients with HCC within TT (53.56 ± 10.56), AT (84.76 ± 11.32), and TF (48.33 ± 7.79) samples. As determined with a paired t test ($n = 18$), AC133-MVD in AT samples was the highest among TF, AT, and TT samples, whereas CD34-MVD (microvessels/0.74 mm^2) in TT samples was the highest among TF (86.51 ± 25.10), AT (117.32 ± 37.81), and TT (323.29 ± 101.14) samples (Fig. 5A and B).

Compared with amounts in HC ($n = 8$), the relative levels of *AC133* and *CD34* gene expression were elevated in TF, AT, and TT samples ($n = 64$; Fig. 5C and D). As determined with a paired t test, the relative level of *AC133* gene expression in AT samples was the highest (Fig. 5C), whereas the relative *CD34* gene expression in TT samples was the highest among TF, AT, and TT samples (Fig. 5D).

As determined by protein and gene expression levels, $AC133^+$ EPCs in patients with HCC were recruited and incorporated into the microvessels in TF, AT, and TT and were especially abundant in AT.

$VEGF_{165}$ and PDGF-BB in plasma and liver of HCC for mobilization and recruitment of EPCs. The median level of preoperative plasma $VEGF_{165}$ (223.80 ± 32.19 pg/mL) and PDGF-BB (605.25 ± 128.38 pg/mL) in patients with HCC ($n = 20$) was higher than that of plasma $VEGF_{165}$ (24.79 ± 4.88 pg/mL; $P = 0.003$) and PDGF-BB (37.13 ± 10.46 pg/mL; $P = 0.000$) in HC ($n = 15$; Fig. 6A). In the peripheral blood of patients with HCC, the relative level of *AC133* gene expression correlated with plasma VEGF levels ($r = 0.629$; $P = 0.009$) and PDGF-BB ($r = 0.618$; $P = 0.024$; Fig. 6B). Thus, $VEGF_{165}$ and PDGF-BB in plasma may be key factors for the mobilization of bone marrow-EPCs into peripheral blood. In tissues, the relative concentration of $VEGF_{165}$ ($P = 0.017$) and PDGF-BB ($P = 0.001$) in HC ($n = 4$) was the lowest of the four groups. The relative concentration of $VEGF_{165}$ was lower in TT than in TF ($P = 0.025$) and AT ($P = 0.024$), whereas the relative concentration of PDGF-BB was higher in TT than that in TF ($P = 0.004$) and AT ($n = 14$; $P = 0.006$; Fig. 6C). Furthermore, there was no correlation between the relative concentration of the two factors ($VEGF_{165}$ and PDGF-BB) and the relative levels of *AC133* gene expression and MVD in the respective groups. Nevertheless, recruitment and homing of EPCs in the liver with HCC may also be affected by other proangiogenic factors besides $VEGF_{165}$ and PDGF-BB.

Relationship between the level of AC133 gene expression in tissue and clinical variables. Within the analyzed clinical variables (gender, age, total bilirubin, and glucose), the relative level of *AC133* gene expression was distributed equally in TF,

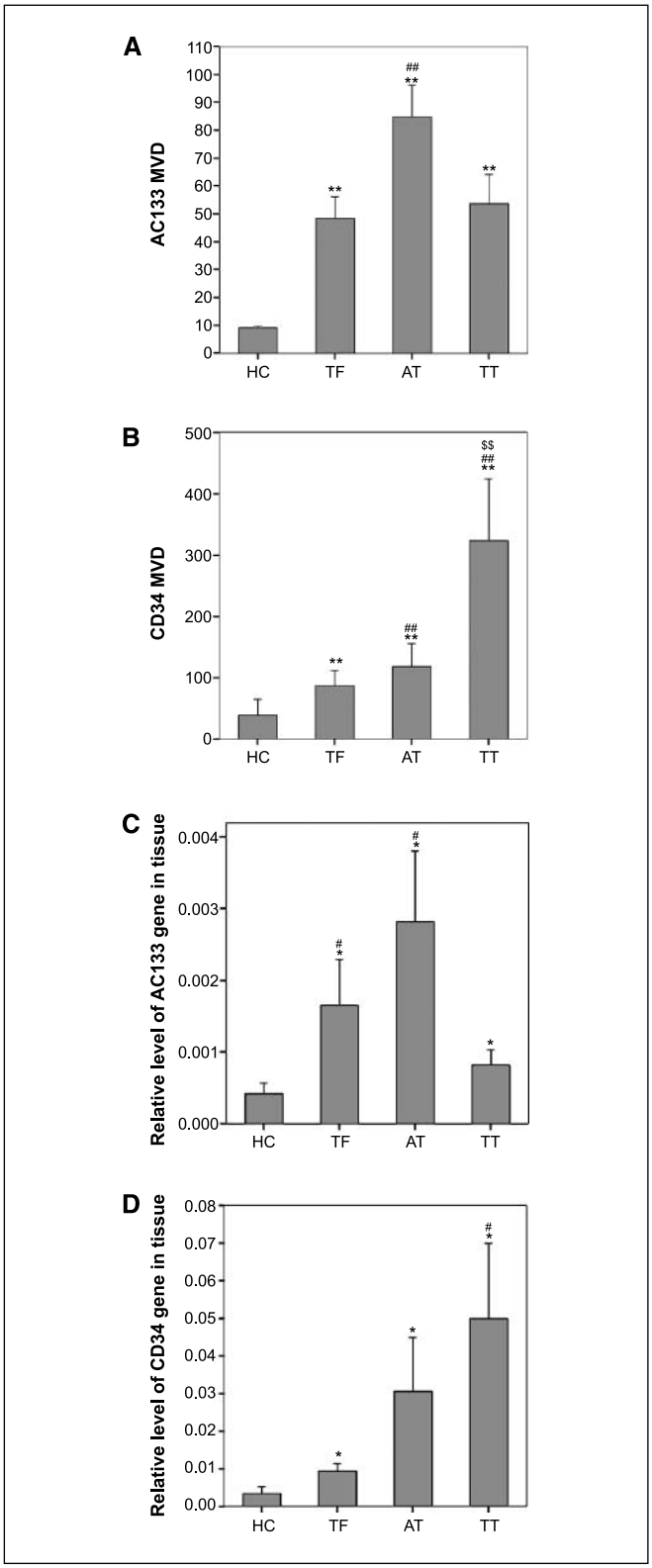

Fig. 5. Frequency of AC133 and CD34 antigens and genes in HC, TT, AT, and TF. *A,* AC133-MVD in HC ($n = 4$), TF, AT, and TT ($n = 18$) were compared. **, $P < 0.01$ versus HC; ##, $P < 0.01$ versus TF and TT. *B,* CD34-MVD in HC ($n = 4$), TF, AT, and TT ($n = 18$) were compared. **, $P < 0.01$ versus HC; ##, $P < 0.01$ versus TF; $$, $P < 0.01$ versus AT. *C,* relative level of *AC133* gene in HC ($n = 8$), TF, AT, and TT ($n = 64$). *, $P < 0.05$ versus HC; #, $P < 0.05$ versus TT. *D,* relative level of *CD34* gene in HC ($n = 8$), TF, AT, and TT ($n = 64$). *, $P < 0.05$ versus HC; #, $P < 0.05$ versus TF. Columns, mean; bars, SD.

AT, and TT samples. Clinicopathologic variables that correlated with the relative level of *AC133* gene expression in AT samples included platelets ($\leq 150 \times 10^9$/L or $>150 \times 10^9$/L), hepatitis B virus status, the absence of tumor capsule, venous invasion, positive frequency of PCNA (1 and 2), and early recurrence time (≤6 or >6 months). Furthermore, the relative level of *AC133* gene expression in TF sample correlated only with platelets and hepatic vein invasion, whereas the relative level of *AC133* gene expression in TT sample correlated with capsule invasion. Therefore, the level of *AC133* gene expression in AT could be used as a biomarker for predicting the progression of HCC (Table 1).

Discussion

The previous reports have indicated that EPCs can be identified by simultaneous expression of the cell surface markers CD34, AC133, and kinase insert domain-containing receptor (4, 5). In this investigation, $AC133^+$ and $CD34^+$ cells were regarded as EPCs. This is supported by the following: (*a*) AC133, the surface marker of progenitor cells, is used to evaluate the level of EPCs in circulation or in tumors such as non–small cell lung cancer (9), HCC (22), breast cancer (35), and multiple myeloma (36). (*b*) CD34-MVD has been recognized as a biomarker for tumor angiogenesis, especially in HCC. (*c*) Kinase insert domain-containing receptor is expressed not only in hepatocytes and liver cancer cells but also in endothelial cells and progenitor cells in HCC with cirrhosis present (37). In addition, kinase insert domain-containing receptor expression in liver with HCC (TT, AT, and TF) was diffuse and similar in levels.[4] A possible explanation for this observation might be that kinase insert domain-containing receptor staining is not specific to endothelial cells. (*d*) It is conceivable that $AC133^+$ cells are cancer stem cells in HCC (38), prostate cancer (39), and colon cancer (40) because they possess a marked capacity for proliferation, self-renewal, and differentiation, although they represent only a small population (~0.1-2.5%) of the cancer cells (39, 40). However, there have not been any reports that $AC133^+$ and $CD34^+$ cells are cancer stem cells. In fact, in the present investigation, $AC133^+$ and $CD34^+$ cells, which were present in the vessel walls, were classified as endothelial-like cells, not as cancer cells, by our senior pathologist following immunohistochemistry and immunofluorescent staining. Further investigation showed that $AC133^+$ cells were negative for hepatocyte paraffin-1 antigen, which is the specific marker of hepatocytes or cancer cells in HCC.[5]

Thus far, the clinical significance of circulating EPCs has been considered for non–small cell lung cancer (9), HCC (22), and breast cancer (35). Especially in breast cancer, a surrogate biomarker approach involving measurement of circulating EPCs has been used to determine the optimal dose of antiangiogenic drugs (35). In the current investigation, the relative levels of *AC133* gene and antigen expression were higher in the peripheral blood of patients with HCC compared with HC, which positively correlated with plasma $VEGF_{165}$, PDGF-BB, and some clinicopathologic variables.[5] In Poon et al. study, CFU scores, which correlated with the levels of VEGF and interleukin-8, were elevated in patients with unresectable HCC compared with patients with early resectable HCC or liver cirrhosis and HC (22). Thus, EPCs were mobilized from bone marrow in HCC patients. The surface marker AC133 and CFU scores may be valuable biomarkers to predict progression of HCC.

It is generally accepted that vascularization of tumors arises exclusively from endothelial sprouting. Arbab et al. (6) and Shirakawa et al. (7) used mouse tumor models to show that bone marrow–derived EPCs are also involved in tumor vasculogenesis, especially in the surrounding of the tumor. Until recently, there have been only two clinical reports that have evaluated the participation of EPCs in the progression of solid

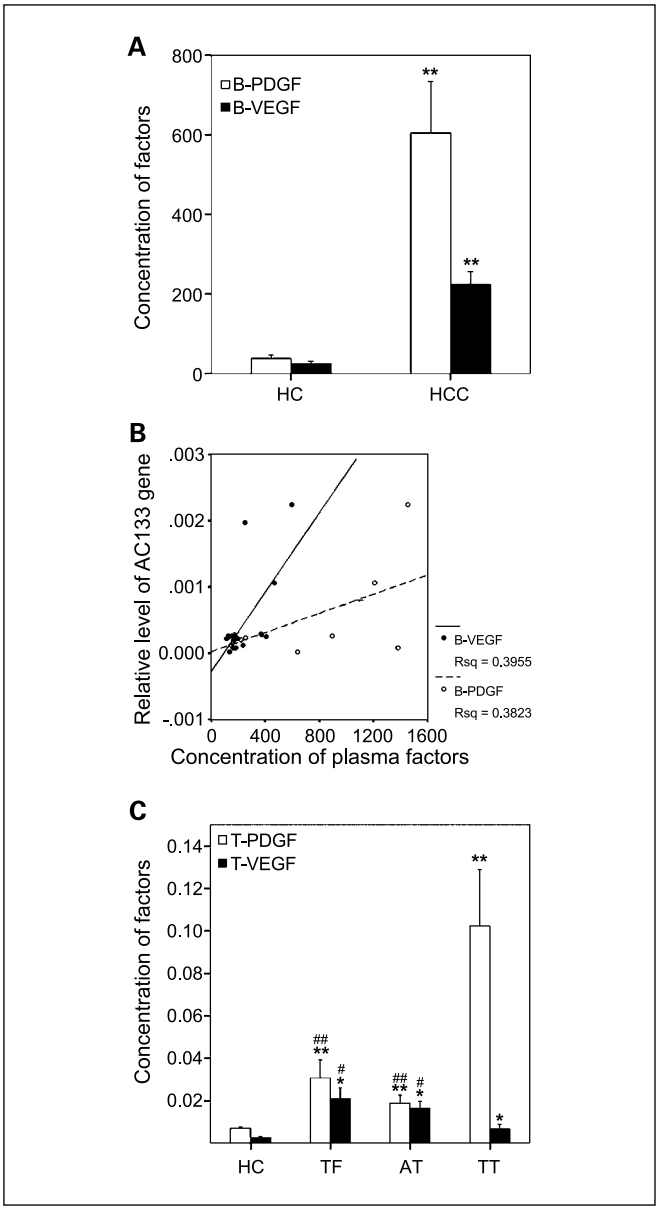

Fig. 6. Concentration of $VEGF_{165}$ and PDGF-BB in plasma and tissue. *A,* plasma $VEGF_{165}$ and PDGF-BB level in the HC ($n = 15$) and patients with HCC ($n = 20$). **, $P < 0.01$. *B,* correlation of the relative level of *AC133* gene with plasma $VEGF_{165}$ and PDGF-BB level in peripheral blood of patients with HCC ($n = 12$). *C,* the relative concentration of $VEGF_{165}$ and PDGF-BB in HC ($n = 4$), TF, AT, and TT ($n = 14$). *, $P < 0.05$; **, $P < 0.01$ versus HC; #, $P < 0.05$; ##, $P < 0.01$ versus TT. Columns and points, mean; bars, SD.

[4] Unpublished data.
[5] Data unpublished due to limitations in manuscript length.

tumors (8, 9). In the current investigation, EPCs were incorporated into vessel walls of different sizes, mostly in the microvessels in cirrhotic and tumor tissues of patients with HCC, although only a few EPCs were found in the portal area vessels in normal liver samples. Proangiogenic factors, such as VEGF (41) and PDGF (42), which have the strong expression in HCC and cirrhotic tissue samples, may regulate not only endothelial cell proliferation but also promote EPC mobilization and homing into HCC and cirrhotic tissue. Furthermore, tumor stroma provides pathways for neovessels and serves as a reservoir for growth factors and other macromolecules (43). Our results agree with this concept. We found that encapsulated HCCs have more EPCs in AT and TT than nonencapsulated ones. In these tumors with capsule invasion, more EPCs were present in tumor tissue. Of note, a higher level of $VEGF_{165}$ mRNA in nonmalignant liver tissue correlated significantly with a higher risk of HCC recurrence and recurrence-related mortality, vascular permeation, daughter nodules, cellular dedifferentiation, and absent or incomplete capsule (44). In present investigation, the relative level of *AC133* gene expression in AT also correlated with clinicopathologic variables (platelets, HBsAg status, the absence of tumor capsule, venous invasion, positive frequency of PCNA, and early recurrence time). Therefore, mobilized EPCs participate in tumor vasculogenesis of HCC. *AC133* gene or antigen in AT may be used as an angiogenic biomarker predicting the progression of HCC.

Thus far, there are no published investigations of the enrichment of EPCs in nonmalignant liver tissue, especially in tissue adjacent to tumors. The basis for the presence of more EPCs in microvessels in tissue adjacent to tumors is not known. The recruitment and homing of EPCs are affected by hypoxia, angiogenic factors, and adherent molecules (45). In this regard, the particular pathology in HCC with liver cirrhosis may be important.

HCC is a cancer associated in most cases with chronic liver disease, such as chronic viral hepatitis and cirrhosis, especially in Southeast Asia. The nonmalignant liver itself has a precancerous change with angiogenesis. During liver cirrhosis, fibrogenesis induces intrahepatic shunts and a barrier between the sinusoids and the hepatocytes (46). In addition, hepatitis B

Table 1. The relationship between the relative level of *AC133* gene in TF, AT, and TT and clinical variables

Clinical variables	Class	No.	*AC133* in TF		*AC133* in AT		*AC133* in TT	
			Mean	*P*	Mean	*P*	Mean	*P*
Platelets (10^9/L)	<150	41	0.00139	0.002*	0.00163	0.003*	0.00072	0.385
	≥150	23	0.00529		0.00504		0.00010	
HBV infection	No	8	0.00362	0.328	0.00746	0.001*	0.00041	0.234
	Yes	56	0.00267		0.00207		0.00088	
AFP	<363	30	0.00239	0.370	0.00331	0.533	0.00066	0.271
	≥363	34	0.00314		0.00235		0.00096	
Cirrhosis	No	4	0.00106	0.528	0.00004	0.388	0.00142	0.084
	Yes	60	0.00291		0.00295		0.00078	
TNM classification	I-II	37	0.00279	0.344	0.00357	0.827	0.00093	0.840
	III-IV	27	0.00400		0.00392		0.00101	
Tumor size (cm)	<2	7	0.00106	0.798	0.00106	0.738	0.00020	0.450
	≥2, <5	17	0.00276		0.00256		0.00114	
	≥5	40	0.00311		0.00333		0.00079	
Lesion	Solitary	40	0.00219	0.292	0.00325	0.184	0.00084	0.492
	Multiple	24	0.00379		0.00181		0.00079	
Node metastasis	No	55	0.00197	0.418	0.00289	0.570	0.00069	0.226
	Yes	9	0.00130		0.00180		0.00061	
Necrosis in mass	No	35	0.00330	0.157	0.00301	0.260	0.00074	0.736
	Yes	29	0.00218		0.00255		0.00091	
Portal vein invasion	No	40	0.00253	0.841	0.00106	0.003*	0.00097	0.071
	Yes	24	0.00322		0.00576		0.00056	
Hepatic vein invasion	No	42	0.00130	0.002*	0.00143	0.000*	0.00065	0.082
	Yes	22	0.00563		0.00602		0.00113	
Hepatic capsule invasion	No	29	0.00150	0.064	0.00162	0.061	0.00073	0.679
	Yes	35	0.00386		0.00396		0.00089	
Capsule invasion	No	43	0.00200	0.142	0.00251	0.588	0.00061	0.024 †
	Yes	21	0.00458		0.00363		0.00131	
Tumor capsule	No	21	0.00117	0.096	0.00082	0.022 †	0.00070	0.460
	Yes	43	0.00366		0.00429		0.00094	
Differentiation	Low	13	0.00125	0.166	0.00092	0.221	0.00064	0.494
	Mid-high	51	0.00330		0.00272		0.00090	
PCNA (intensity)	1	23	0.00401	0.077	0.00071	0.035 †	0.00079	0.733
	2	41	0.00211		0.00383		0.00083	
Recurrence time (mos)	<6	18	0.00311	0.921	0.00615	0.000*	0.00090	0.059
	≥6	46	0.00267		0.00137		0.00061	

Abbreviations: HBV, hepatitis B virus; AFP, α-fetoprotein; TNM, tumor-node-metastasis.
*$P < 0.01$.
†$P < 0.05$.

virus X protein increases the transcriptional activity and protein level of hypoxia-inducible factor-1α, thereby promoting angiogenesis during hepatocarcinogenesis (47). Regeneration in the cirrhotic liver would pose a potential of malignant degeneration and correlated with serum VEGF level (48). Moreover, VEGF expression is also modulated by inflammatory cytokines released from infiltrating inflammatory cells in surrounding cirrhotic liver tissues (25). It is developing into an angiogenetic environment that may secrete higher proangiogenic factors. More and more investigations reported proangiogenic factors, such as VEGF (25), hepatic growth factor (26), and inducible nitric oxide (49), have higher expression in the surrounding liver than in tumors, which were consistent with our results. In addition, our further investigations have indicated that the expression of CD105, hypoxia-inducible factor-1α, and vascular cell adhesion molecule-1 are elevated in AT compared with TF and TT (parts of data to be published in another article about the distribution of CD105 in HCC). So it is concluded that the recruitment and homing of EPCs into AT may be affected by hypoxia-inducible factor-1α, proangiogenic factors, and cell-matrix adhesion molecules resulting from both liver cirrhosis and HCC. The exact mechanism on recruitment and homing of EPCs into liver cirrhosis and cancer is worthy of further investigation.

In summary, our data indicate that (*a*) EPCs were mobilized into the peripheral blood of patients with HCC, and this mobilization correlated with plasma $VEGF_{165}$ and PDGF-BB; (*b*) EPCs were incorporated into vessel walls of different sizes and were found primarily in the microvessels in cirrhotic and malignant liver specimens; (*c*) the relative level of *AC133* gene expression in AT correlated with clinicopathologic variables, such as platelets ($\leq 150 \times 10^9$/L or $>150 \times 10^9$/L), hepatitis B virus status, the absence of a tumor capsule, hepatic or portal vein invasion, positive frequency of PCNA (1 and 2), and early recurrence time (≤6 or >6 months). These findings suggest that mobilized EPCs participate in the vasculogenesis of HCC and may serve as biomarkers for predicting the progression of HCC. As EPCs are endowed with the capacity to home the tumor vasculature, they might be used to deliver drugs. In addition, the recruitment and distribution of EPCs in HCC was different from other cancers. Therefore, the identification of chemokines/cytokines and tissue-specific extracellular matrix components that are involved in the recruitment of EPCs in HCC might provide new targets for the treatment of HCC.

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Decai Yu, Xitai Sun, Yudong Qiu, et al.

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