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Increased number and function of endothelial progenitor cells in breast cancer patients and the linear correlation with VEGF level

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The number of circulating endothelial progenitor cells (EPCs) was found to increase in patients with breast cancer, but the alteration in EPC function remains to be elusive. We conducted this study to evaluate the number and function of peripheral EPCs of breast cancer patients and its possible underlying mechanism. Besides, the vascular endothelial growth factor (VEGF), VCAM-1, IL-6, and IL-34 levels were measured in blood samples and also *in vitro* in a medium of EPCs. We found that the number of circulating EPCs in breast cancer patients was significantly higher than that in normal control and remarkably augmented in a stage-dependent manner. Meanwhile, a similar enhancement was observed in the migratory, proliferative, and adhesive activity of circulating EPCs originating from breast cancer patients. More importantly, the VEGF level in blood samples was dramatically elevated in comparison to the control, which was correlated positively with the number and activity of circulating EPCs from breast cancer patients. Moreover, *in vitro* medium of EPCs from breast cancer patients highly expressed VEGF compared with that from the control, which also had a positive correlation with the number and activity of circulating EPCs from breast cancer patients. This is the first time to demonstrate that the number and function of circulating EPCs are promoted in breast cancer patients, which are positively related to an enhanced VEGF production. These may provide a novel target for improving the outcome of breast cancer.

Key words: endothelial progenitor cells, breast cancer, vascular endothelial growth factor

Breast cancer is one of the most common malignant tumors in women [1], but the mechanism underlying the development and migration of breast cancer remains to be elusive. The growth of malignant tumors depends on new blood vessels to provide oxygen and micronutrients. Accumulating evidences indicate that tumor-associated neovasculature contributes to the development and metastatic spread of solid malignancies. For example, in breast cancer, angiogenesis is found to facilitate growth and metastasis [2, 3, 4].

Circulating EPCs, derived from hemangioblasts, have the ability to differentiate into mature endothelial cells (ECs) and integrate into nascent neovessels [5]. Under the tumor microenvironment, EPCs are mobilized from bone marrow and home to the tumor site in response to tumor-released cytokines. A previous study suggested that EPCs promoted the growth and progression of malignant tumors via enhancing neovascularization [3]. Others showed that blocking EPCs mobilization resulted in decreased angiogenesis and impaired lung cancer progression [6]. An increased number of EPCs was observed in different types of cancer, including breast cancer or lung cancer, suggesting that EPCs-mediated angiogenesis may promote the development of malignant neoplasm [4, 5, 7].

Various cytokines and growth factors are involved in the mobilization and homing of EPCs, including vascular endothelial growth factor (VEGF), VCAM-1, IL-6, and IL-34 [6]. EPCs are proved to regulate the process of new vessel formation via secreting a series of cytokines [8]. One of the most widely studied cytokines is VEGF, which is highly expressed in different types of malignant tumors [9]. VEGF has multiple effects on vascular endothelial cells, such as increasing vascular permeability, reshaping the extracellular

matrix of malignant tumor cells to support the adhesion of tumor tissue, or reducing cell apoptosis to improve survival [10, 11]. A higher level of VEGF was found in breast cancer patients, which could augment the number and activity of EPCs [12]. Therefore, we propose that VEGF may contribute to the growth and progression of breast cancer by affecting the number and function of EPCs. To verify our proposal, we conduct a pilot study to investigate the number and function of EPCs in patients with breast cancer.

Patients and methods

Subject characteristics. Blood samples were collected from twenty healthy women, twenty female patients with pathologically diagnosed breast cancer whose clinical subtype is ER-positive/Her2 negative. Patients with a history of autoimmune disease, mental disease, diabetes, hypohepatia, renal insufficiency, malignant tumor, gestation period, breastfeeding, or those unwilling to accept the test subjects were excluded. Table 1 shows the baseline characteristics of the two subject groups. Blood samples were collected for measuring EPCs, fasting plasma glucose, aspartate amino transferals, alanine transaminase, blood urea nitrogen, triglycerides, high-density lipoprotein cholesterol, LDL-cholesterol, plasma glucose, serum creatinine**,** high-density lipoprotein, total cholesterol, estradiol, and so on. Table 2 shows the characteristics of the stage of breast cancer patients. The study was approved by the Ethical Committee of The First Affiliated Hospital of Sun Yat-sen University.

Isolation and cultivation of EPCs. EPCs were isolated and cultured as previously described [13–19]. In brief, being isolated from two groups by Ficoll density-gradient centrifugation, peripheral blood mononuclear cells (PBMNCs) were cultured in endothelial cell basal medium-2 (EBM-2, Clonetics). After culturing for four days, the non-adherent cells were removed by thoroughly washing with EBM-2 and the remaining cells were quantitatively identified as EPCs.

Quantitative determination of EPCs. EPCs were quantitatively measured with two methods as previously described [19, 20]. First, flow cytometry analysis (Beckman Coulter, Fullerton, CA, USA) was used to evaluate the number of circulating EPCs. Briefly, 100 ml peripheral blood was immunostained with human CD34 antibody (Becton Dickinson, Franklin Lakes, NJ, USA) and human KDR antibody (Sigma, USA), followed by a PE-conjugated secondary antibody. After incubation, cells were lysed, washed with PBS, and fixed in 4% paraformaldehyde after removing debris and platelets. The number of circulating EPCs was assessed by the ratio of CD34+/KDR+ cells per 100 PBMNCs. Second, a cell culture assay was used to evaluate the number of circulating EPCs. PBMNCs of blood samples were isolated by Ficoll density-gradient centrifugation and suspended into endothelial cell basal medium supplemented with EGM-2 MV. Then, PBMNCs were incubated at 37 °C in a humidified environment with 5% CO_2. The cells were observed by a phase-contrast microscope. Then, the EPCs were quantified using 1,1'-dioctadecy-3,3,3',3'-tetramethylindo-carbocyanine perchlorate-labeled acetylated LDL (DiI-acLDL) uptake and FITC-labeled Ulex europeus agglutin (lectin) staining. The EPC number was measured by counting DiI-acLDL/lectin double-positive cells/ ×200 with two independent observers blinded to the study.

Proliferation, migration, and adhesion of EPCs *in vitro*. Proliferation, migration, and adhesion of EPCs *in vitro* were determined as in the previous studies [19–21]. Briefly, EPC proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After culturing for seven days, EPCs were digested with 0.25% trypsin and then cultured in a serum-free medium in 96-well culture plates (200 ml/well). After culturing for 48 h, EPCs were supplemented with 10 ml MTT (Fluka Co. Milwaukee, WI, USA) and incubated for 6 h. The supernatant was removed and EPCs were mixed with 200 ml dimethyl sulfoxide (DMSO) for 10 min before the OD value was obtained at 490 nm.

EPC migration was evaluated by using a modified Boyden chamber. EPCs were harvested by centrifugation, and then resuspended in an endothelial basal medium. 2×10^4 endothelial progenitor cells were kept in the upper chamber of a modified Boyden chamber. The chamber was put in a 24-well culture dish containing an endothelial basal medium and human vascular endothelial growth factor (50 μg/ml). After incubation for 72 h, the lower side of the filter was washed with phosphate-buffered saline (PBS) and fixed with 2%

Table 1. Clinical and biochemical characteristics.

Characteristics	Breast cancer (n=20)	Control (n=20)
Age (years)	44.5±8.6	41.9±7.1
BMI (kg/cm^2)	22.5±2.1	23.2±1.8
Systolic blood pressure (mmHg)	108.6±9.1	112.5±8.6
Diastolic blood pressure (mmHg)	68.3±5.4	67.5±6.2
Heart rate (beats/min)	80.6±7.0	79.5±5.9
AST (mmol/l)	24.6±7.3	23.4±6.2
ALT (mmol/l)	20.8±6.1	19.6±5.7
BUN (mmol/l)	5.5±1.1	5.7±0.8
Cr (mmol/l)	65.6±11.0	60.7±10.6
LDL (mmol/l)	2.73±0.31	2.80±0.36
TC (mmol/l)	4.50±0.64	4.3±0.53
HDL (mmol/l)	0.35±0.2	0.40±0.3
TG (mmol/l)	1.50±0.2	1.59±0.23
FPG (mmol/l)	4.36±0.54	4.60±0.51
Estradiol (pmol/l)	98.5±26.4	101.6±24.6

Notes: Data are given as mean ± SD. Abbreviations: BMI-body mass index; AST-aspartate amino transferals; ALT-alanine transaminase; BUN-blood urea nitrogen; Cr-serum creatinine; LDL-low-density lipoprotein; TC-total cholesterol; HDL-high density lipoprotein; TG-triglyceride; FPG-fasting plasma glucose

Table 2. Clinical and biochemical characteristics.

Characteristics	Stage I (n=4)	Stage II (n=6)	Stage III (n=6)	Stage IV (n=4)
Median age (years)	39.5 (range 26–48)	46 (range 29–60)	44.5 (range 34–51)	50.5 (range 47–54)
Tumor size (T)				
Tis	2	N/A	N/A	N/A
T1	2	3	1	N/A
T2	N/A	3	3	1
T3	N/A	N/A*	2	3
Lymph node status (N)				
N0	4	2	N/A	N/A
N1	N/A	3	2	2
N2	N/A	1	3	2
N3	N/A	N/A	1	
Sites of metastasis				
1 site	N/A	N/A	N/A	1
2 sites	N/A	N/A	N/A	2
≥3 sites	N/A	N/A	N/A	1
CD34+/KDR+ cells (%)	0.029±0.0075	0.049±0.0[1]0a	0.058±0.0[14]ab	0.064±0.01[2]ab
acLDL/lectin positive cells/×200	51.2±8.1	65.3±9.6[a]	78±7.5[ab]	84.5±6.9[ab]

Notes: *Not applicable; [a]p<0.05 vs. stage I; [b]p<0.05 vs. stage II

paraformaldehyde. Cell nuclei were stained with DAPI for counting the number of EPCs. Cells migrating in the lower chamber were assessed manually and microscopically.

EPC adhesion was detected with plate coated with fibronectin. 2×10^4 cells in each well of a 24-well plate were treated with or without SDF-1 (100 ng/ml) for 5 h at 37 °C. Non-attached cells were disregarded with PBS, and adherent EPCs were fixed with 4% paraformaldehyde and stained with 0.3% crystal violet. The adherent EPCs were counted.

Measurement of VEGF, VCAM-1, IL-6, and IL-34 levels in blood samples and *in vitro* medium of isolated EPCs. Plasma levels of VEGF, VCAM-1, IL-6, and IL-34 and *in vitro* medium of isolated EPCs were determined by the Quantikine ELISA kit (R&D Systems, Minneapolis, USA) as previously described [22] according to the manufacturer's instructions.

Statistical analysis. The statistical software was SPSS V20.0 (SPSS Inc., Chicago, Illinois). All data were presented as mean values ± SD. Comparisons between the two groups were analyzed by t-test. Univariate correlations were calculated using Pearson's coefficient (r) if data conform to normal distribution or Spearman's coefficient (r) if non-normal distribution. Statistical significance was assumed if a null hypothesis could be rejected at $p<0.05$.

Results

The baseline of clinical characteristics. As shown in Table 1, the baseline characteristics of the age, BMI, systolic blood pressure, diastolic blood pressure had no statistical difference between breast cancer and normal control group ($p>0.05$), and the hepatorenal function indicators (AST, ALT, BUN, Cr, FPG), blood lipid level (LDL, TC, HDL, TG), and estradiol did not differ between the two groups ($p>0.05$).

Demographics and tumor characteristics of the included breast cancer patients are summarized in Table 2. The clinical classifications and staging of each patient were evaluated using the Revision of the American Joint Committee on Cancer Staging System for Breast Cancer. The number of circulating EPCs was assessed by FACS analysis and a phase-contrast fluorescent microscope. The baseline of circulating EPCs level in stage II, III, and IV breast cancer patients were significantly higher than that in stage I patients ($p<0.05$). and the baseline of circuiting EPCs levels in stage III and IV patients were elevated more than stage II patients ($p<0.05$). However, there was no significant difference in circulating EPCs level between stage III and IV ($p>0.05$).

The number and activity of circulating EPCs in two groups. As shown in Figure 1, the number of circulating EPCs evaluated by FACS analysis in breast cancer was increased more than that in normal control (Figure 1A). A similar result was confirmed by using double staining acLDL+/lectin+ cells observed in a phase-contrast fluorescent microscope (Figure 1B). The increased number of EPCs urged us to investigate the function of EPCs in breast cancer. As shown in Figure 2, the migratory, proliferative, and adhesive functions of circulating EPCs in breast cancer patients were more active than that in normal control (Figures 2A–2C; Supplementary Figures S1A, S1B). It suggests that circulating EPCs in breast cancer patients have hyperfunction over those in normal people.

Plasma VEGF, VCAM-1, IL-6, and IL-34 levels in two groups. As shown in Figure 3, the plasma VEGF level in breast cancer patients was significantly elevated more than

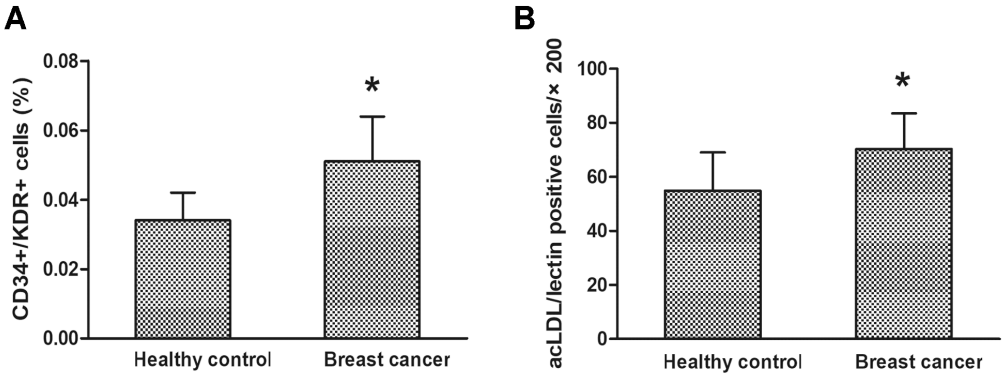

Figure 1. The number of circulating EPCs in the two groups was shown as follows. Evaluated by A) FACS analysis and B) phase-contrast fluorescent microscopy, the number of circulating EPCs in breast cancer was upregulated than those in normal control. Data are given as mean ± SD *vs. normal weight.

that in the control group (Figure 3A). However, there were no significant statistical differences in plasma VCAM-1, IL-6, and IL-34 levels between the two groups (Figures 3B–3D), suggesting that interleukins may have little relationship with the number and function of EPCs in breast cancer. It indicated that plasma VEGF may have an uncharted effect in breast cancer patients.

VEGF, VCAM-1, IL-6, and IL-34 in *in vitro* medium of isolated EPCs in two groups. As shown in Figure 4, the VEGF level secreted by EPCs collected in breast cancer patients was remarkably escalated, more than that in control (Figure 4A). Nevertheless, there were no significant statistical differences in VCAM-1, IL-6, and IL-34 levels secreted by isolated EPCs in two groups (Figures 4B–4D), suggesting that interleukins may not contribute to the increased number and function of EPCs from breast cancer.

The correlation between the **VEGF level in blood samples and the number or function of circulating EPCs** in breast patients. There was a positive correlation between the number of circulating EPCs evaluated by FACS analysis and the plasma VEGF level ($r=0.86$, $p<0.0001$) shown in Figure 5A. Moreover, similar conrrelation between circulating EPCs and plasma VEGF level in breast cancer was confirmed by using double staining acLDL+/lectin+ cells counted in a phase-contrast fluorescent microscope (Figure 5B, $r=0.926$, $p<0.0001$). In addition, the plasma VEGF level was positively related to migratory, proliferative, or adhesive function of EPCs respectively ($r=0.533$, $p<0.0001$; $r=0.782$, $p<0.0001$ and $r=0.712$, $p<0.05$, respectively) shown in Figures 5C–5E. It showed that circulating VEGF level may be associated with hyperfunction of EPCs in breast cancer patients.

The correlation between the *in vitro* medium of isolated EPCs and the number or function of circulating EPCs in breast patients. The number of circulating EPCs was positively correlated with the VEGF level secreted by isolated EPCs ($r=0.844$, $p<0.0001$) shown in Figure 6A. A similar correlation between isolated EPCs and VEGF level

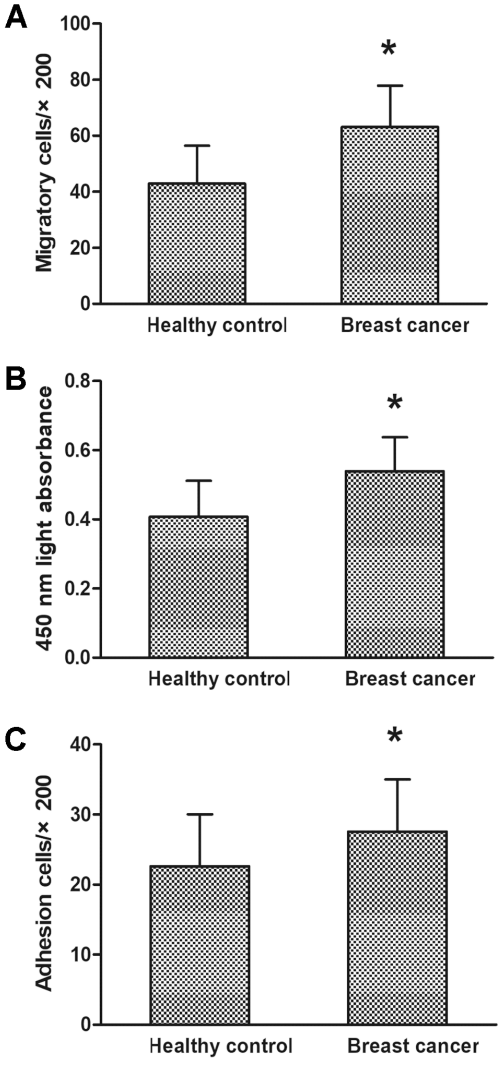

Figure 2. The function of circulating EPCs in the two groups was shown as follows. The migratory (A), proliferative (B), and adhesion (C) activities of circulating EPCs in breast cancer were higher than those in normal control. Data are given as mean ± SD *vs. normal weight.

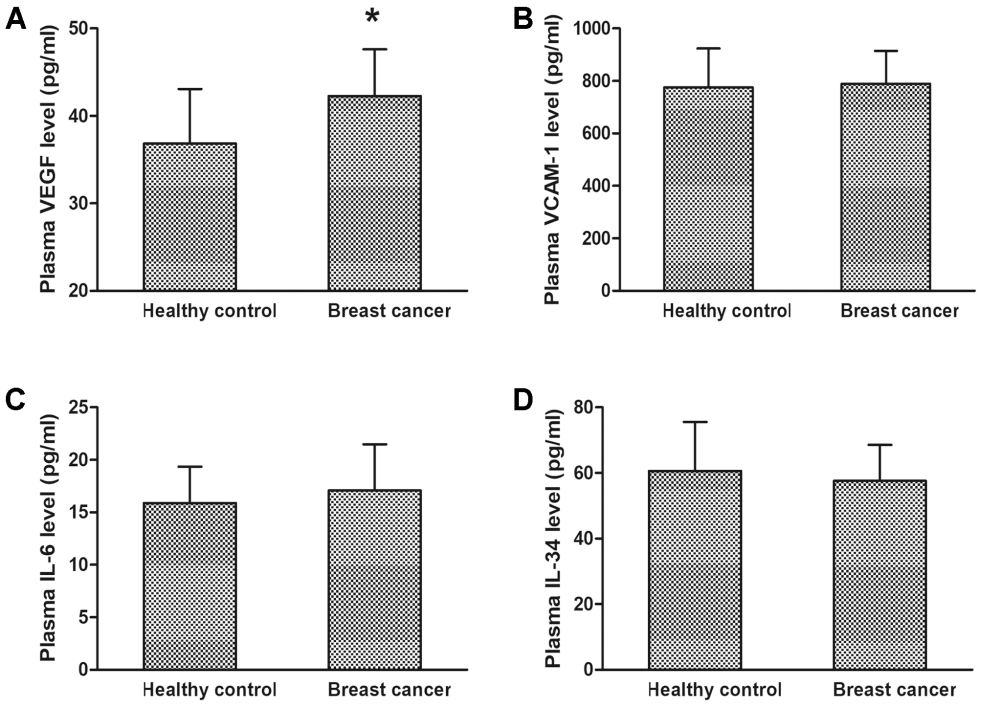

Figure 3. The plasma VEGF, VCAM-1, IL-6, and IL-34 levels in the two groups were shown as follows. A) The plasma VEGF level in breast cancer was increased than that in normal control. B–D) There was no significant difference in the plasma VCAM-1, IL-6, and IL-34 levels between the two groups. Data are given as mean ± SD *vs. normal weight.

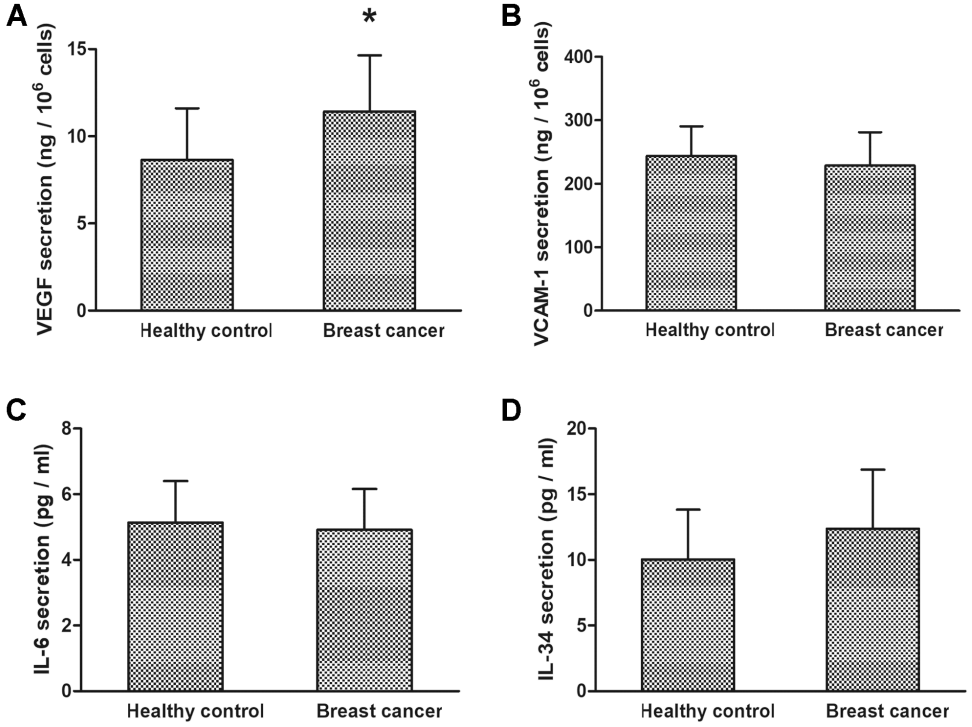

Figure 4. The VEGF, VCAM-1, IL-6, and IL-34 secreted by EPCs in the two groups were shown as follows. A) The VEGF secreted by EPCs in breast cancer was higher than that in normal control. B, C, D) There was no significant difference in VCAM-1, IL-6, and IL-34 secreted by EPCs between the two groups. Data are given as mean ± SD. *vs normal weight.

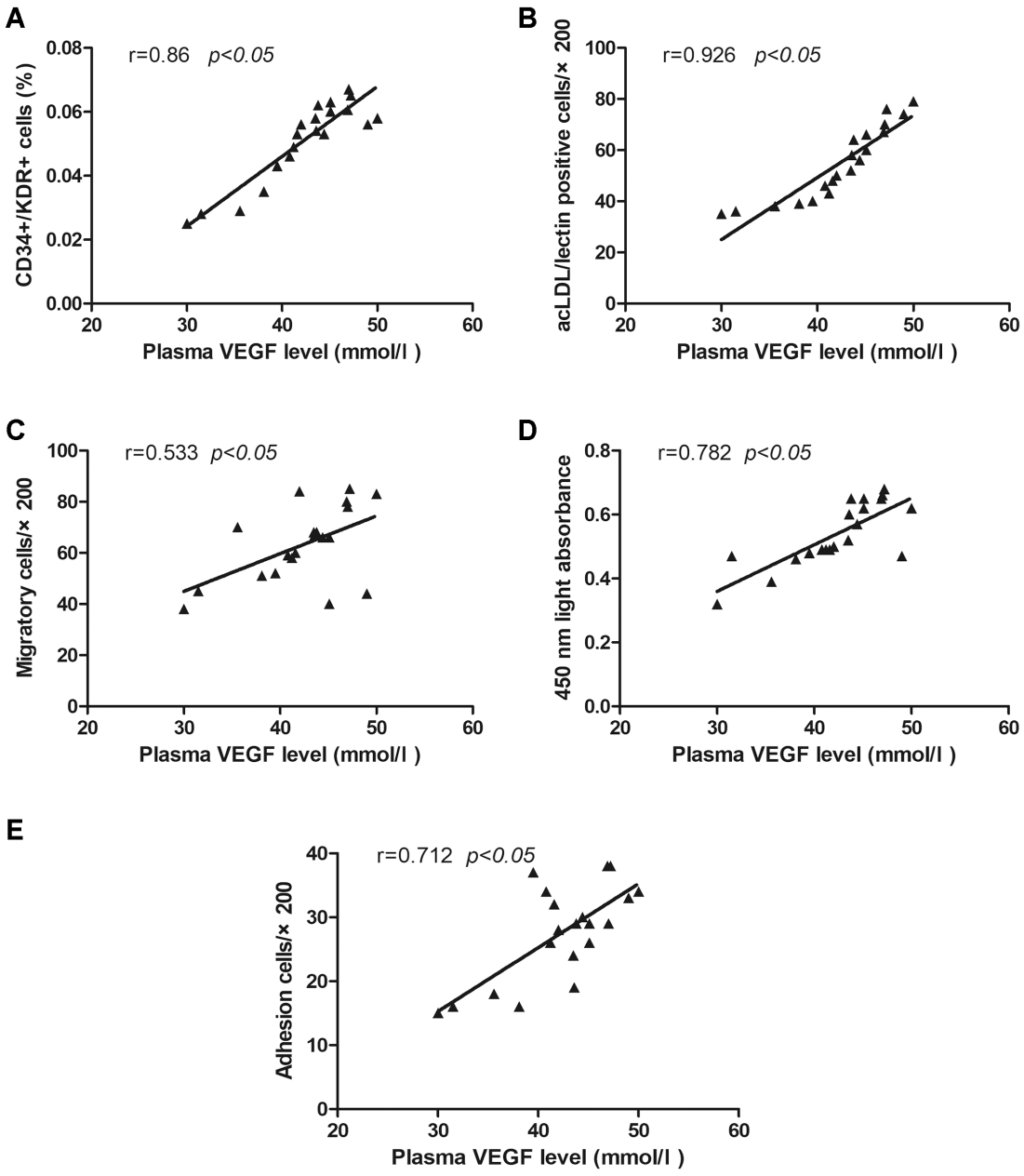

Figure 5. The correlation between the number and function of circulating EPCs with the plasma VEGF level was shown as follows. There was a positive correlation between the number of circulating EPCs, evaluated by A) FACS analysis and B) phase-contrast fluorescent microscopy, and the plasma VEGF level. In addition, the plasma VEGF level was positively related to the EPC migration (C), proliferation (D), or adhesion (E).

of the *in vitro* medium was confirmed by using double staining acLDL+/lectin+ cells counted in a phase-contrast fluorescent microscope (Figure 6B, r=0.897, p<0.0001). Moreover, the VEGF level secreted by isolated EPCs had positive relation to migratory, proliferative, or adhesive function of EPCs, respectively (r=0.457, p=0.043; r=0.809, p<0.0001 and r=0.675, p=0.001, respectively) as shown in Figures 6C–6E. It revealed that the hyperfunction of cultured EPCs isolated from breast cancer patients was relevant to the VEGF level secreted by EPCs.

Discussion

In our study, a markedly higher number and activity of circulating EPCs in the patients with breast cancer was found as compared to the health control. The quantitative and

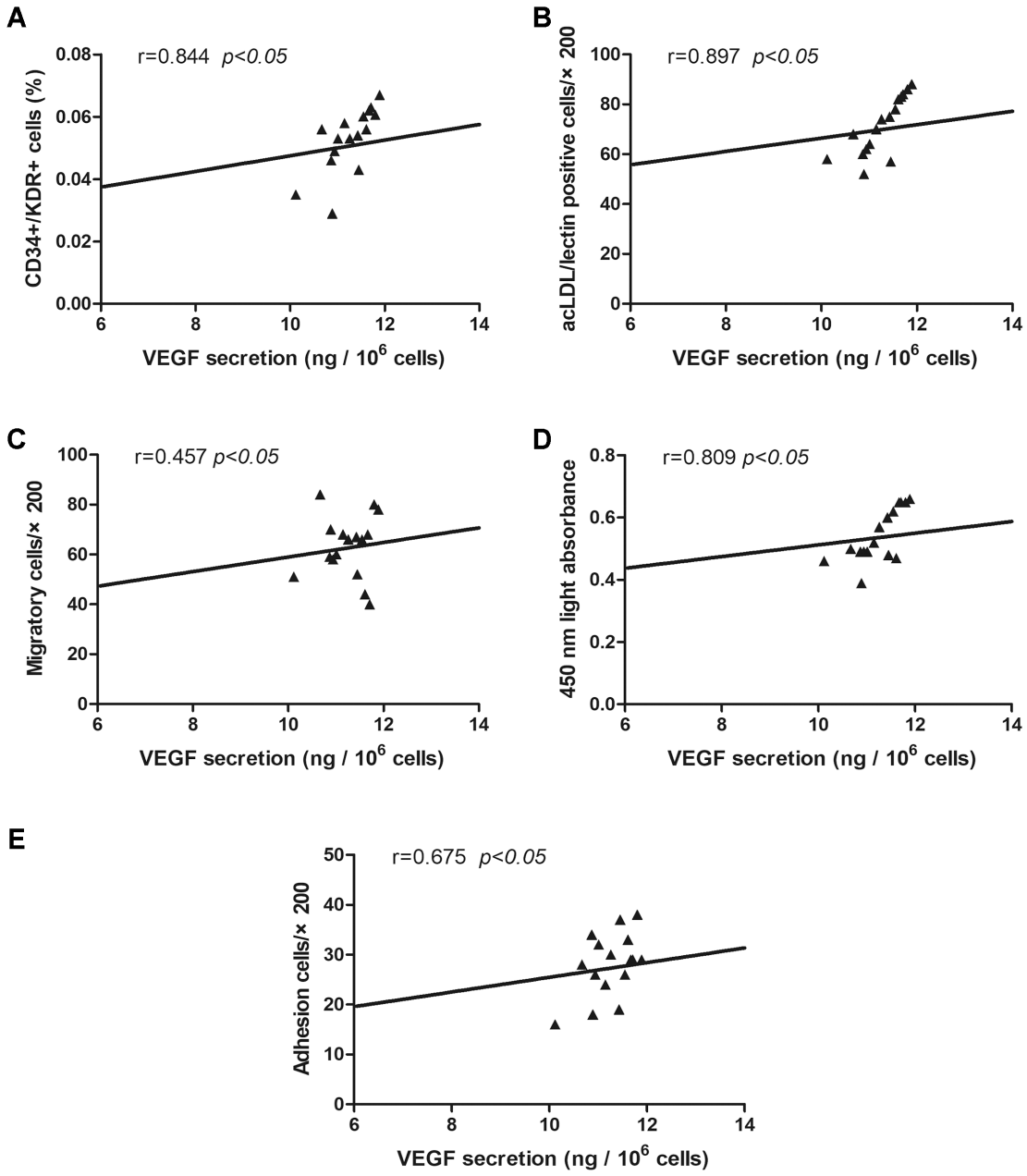

Figure 6. The correlation between the number and function of cultured EPCs and VEGF secretion was shown as follows. There was a positive correlation between the number of circulating EPCs, evaluated by A) FACS analysis and B) phase-contrast fluorescent microscopy, and the VEGF secretion level. In addition, the level of VEGF secretion was positively related to cultured EPC migration (C), proliferation (D), or adhesion (E).

qualitative alterations of circulating EPCs were potentially related to the enhanced VEGF secretion. Our study firstly indicated that a significant association was observed between the number or function of circulating EPCs and VEGF level in blood samples or *in vitro* medium.

The growth and metastatic spread of the majority of tumors are dependent on tumor vascularity [1, 12, 23]. Many studies have suggested that EPCs are involved in tumor-associated vasculogenesis, which in turn drives the development and metastatic progression of several malignant neoplasms [2–7]. In the present study, the antigenic phenotype of EPCs was defined as a co-expression of CD34+ and KDR+ marker identified by the fluorescence-activated cell sorting analysis. Our results confirmed that the number of circulating EPCs was escalated in breast cancer patients and the functions such as migration, proliferation, or adhesion of

EPCs in breast cancer patients were upregulated in comparison to healthy females, suggesting that EPCs probably play a vital role in the development of breast cancer.

Previous studies have shown that tumor cells-producing plasma cytokines could promote the mobilization of EPCs [6, 8, 24–26]. Thus, we hypothesized that some of the cytokines were associated with circulating EPCs. Compared with the healthy control, plasma VEGF level, no ICMA-1, IL-6, and IL-34, was found to be upregulated in the patients with breast cancer. Furthermore, our data showed that plasma VEGF sharply increased migration, proliferation, and adhesion of EPCs. A significantly positive association was confirmed between plasma VEGF and the activity of circulating EPCs. These imply that exogenous VEGF may accelerate the number and activity of circulating EPCs in breast cancer.

EPCs can also secrete a number of paracrine factors to modulate the process of neovessel formation [9, 10, 27, 28]. In our study, EPCs from two groups were isolated and cultured. We found that VEGF in *in vitro* medium was markedly enhanced in patients with breast cancer and then, was positively related to the number and function of circulating EPCs. No similar results were observed in VCAM-1, IL-6, and IL-34 of the *in vitro* medium. These indicated that the change in the number and function of circulating EPCs might be attributable to the VEGF production, independent of both exogenous and endogenous VCAM-1, IL-6, and IL-34. Moreover, targeting VEGF may exert an anti-angiogenic effect by partly interfering with the recruitment of EPCs to the tumor vasculature.

In conclusion, the study firstly demonstrates that the increased number and hyperfunction of circulating EPCs in breast cancer are positively associated with enhanced VEGF production. These may provide us with a novel target for treating and evaluating breast cancer.

Supplementary information is available in the main text of the paper.

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