In order to diagnose the existence of HCC, serum AFP and PIVKA-II levels have been commonly used in clinical practice. However, the sensitivity and specificity of both markers are unsatisfactory for the detection of HCC (12). In the present study, in differentiating HCC from CLD or LC, ROC analysis revealed that the peripheral frequency of TEMs is higher than serum AFP, PIVKA-II, ANG-2 levels with respect to AUC, sensitivity and specificity. Thus, the frequency of TEMs could be better than AFP and PIVKA-II for diagnosing HCC. Evaluation of the clinical value of TEMs in the long-term prognosis of HCC patients is further needed as the observation periods in the current study were a maximum of 3.0 years. During the observation period, no significant difference was detected in such outcome between the TEM^{high} and TEM^{low} groups (Supplementary figure 2).

In summary, TEMs are significantly increased both in the periphery and in the liver of HCC patients and hold diagnostic value for HCC. The frequency of TEMs is well correlated with the degrees of neo-vascularization in HCC tissue. Thus, TEMs would be feasible as a diagnostic cellular marker of HCC potentially reflecting angiogenesis in the liver.

Figure Legends

Fig. 1: Identification and phenotypic analyses of TEMs as $CD14^+CD16^+TIE2^+$ cells in the peripheral blood.

A. PBMC obtained from HCC patients were stained and analyzed by flow cytometry. $CD14^+$ monocytes were divided into two distinct subsets, $CD14^{++}CD16^-$ and $CD14^+CD16^+$ cells. These cells were examined for TIE2 expression. The numbers in the histograms depict the percentages of gated cells. Representative plots from three patients are shown.

B. Frequencies of TIE2 in $CD14^+$ monocytes were compared among two distinct subsets; $CD16^+$ monocytes and $CD16^-$ monocytes, See Fig.1A. The bars indicate mean ± SE of 89 patients. *: $p<0.0001$ by Mann-Whitney nonparametric U test.

C. Western-blot analysis of TIE2 expression in FACS-sorted $CD14^+CD16^+$ and $CD14^{++}CD16^-$ cells from HCC patients. As shown, the bands were TIE2 (140 kDa molecular weight) (the top panels) and those are β-actin (45 kDa molecular weight) (the bottom panels), respectively. The representative results of three series of experiments from 7 HCC patients are shown.

D. TEMs and $TIE2^-$ monocytes in the periphery were gated and analyzed for the expression of various molecules, as indicated in the histogram plots. The filled light gray line, black line and gray line depict the negative control, the expression of relevant markers in TEMs and $TIE2^-$ monocytes,

respectively. The percentage of the marker-positive cells is shown in the histograms. The upper numbers are for TEMs and the lower ones are for TIE2$^-$ monocytes. The representative plots of six series of experiments are shown.

E. Comparative analyses of the expression of CCR4, CCR5, CX3CR1, CD40 and CD86 between TEMs and TIE2$^-$ monocytes, assessed by FACS as described above. The bars indicate mean ± SE of six series of experiments. *: $p<0.05$, **: $p<0.005$, ***: $p<0.001$ by Mann-Whitney non-parametric U test.

Fig. 2: Peripheral frequency of TEMs is increased in patients with HCC, with changes paralleling post therapeutic HCC recurrence.

A. Frequencies of peripheral TEMs in CD14$^+$ monocytes are shown in four groups of HCV-positive patients; HS, CH, LC and HCC, see Table.1. *: $p<0.0001$ by Kruscal-Wallis test with Dunn's multiple comparison test.

B. Frequencies of peripheral TEMs in HCC patients at different clinical TNM stages (early stage; I+II; n=70, advanced stage; III+IV; n=19). n.s., not significant by Mann-Whitney nonparametric U test.

C. In patients who underwent RFA therapy or resection of HCC, the frequencies of TEMs in CD14$^+$ monocytes were examined serially after confirmation of complete ablation or operation to remove

HCC lesions. The bold arrows depict the time point of the RFA therapy or the operation. Both panels depict the frequencies of TEMs in patients before and after the treatment. The left panel shows the results of TEMs in patients without HCC recurrence (n=12) as assessed by CT/MRI examinations, while the right panel shows the results in patients with HCC recurrence (n=5), respectively. *: $p \leq 0.05$ by Paired t-test.

D. In patients with HCC who underwent RFA or the operation, the recurrence-free survival rate after the treatment was compared between those with TEM^{high} (frequency of TEMs $\geq$ 2.75; n=45) and TEM^{low} (frequency of TEMs < 2.75; n=44) using the Kaplan-Meier method, with the log-rank test for comparison. TEM^{high} and TEM^{low}, see Table 2. $p = 0.047$.

Fig. 3: TEMs are observed in perivascular areas of HCC tissue, and their frequency is higher in HCC tissue than in the periphery.

A. Immunofluorescence staining was performed as described in Materials and Methods. The staining for CD14 (green), CD16 (red) and TIE2 (red) identifies $CD14^+TIE2^-$, $CD14^+TIE2^+$, $CD14^+CD16^-$ cells and $CD14^+CD16^+$ monocytes in human liver tissue (blue: nuclei counterstained with Dapi). Representative results of the resected samples obtained from 12 HCC patients are shown. The panels show $CD14^+TIE2^+$ cells (A, B, D) and $CD14^+CD16^+$ cells (C) in the perivascular area of HCC tissue (magnification, 400×), Bold arrows depict $CD14^+TIE2^+$ cells, thin arrows $CD14^+CD16^+$ cells, bold

arrowheads vascular endothelial cells ($TIE2^+CD14^-$) and thin arrowheads $CD14^+TIE2^-$ cells.

B. The frequencies of TEMs in 9 patients with HCC are compared for those in the peripheral blood or in the liver. Liver-infiltrating leukocytes are divided into two distinct groups: leukocytes infiltrating non-tumor tissue (NIL) and tumor-infiltrating leukocytes (TIL). These cells were stained using anti-human CD14, CD16 and TIE2 mAbs. The analyses were performed as described in Materials and Methods. The samples were obtained from nine patients who underwent tumor resection. *: $p<0.05$, **: $p<0.0005$, by Paired t-test.

C. The correlation is analyzed between the frequencies of TEMs in PBMC and those in tumor-infiltrated lymphocytes. Analysis (n=14) was based on Pearson's correlation coefficient. P = 0.003, $R^2 = 0.53$

Fig. 4: The degree of microvessels in HCC is correlated with the frequency of TEMs

A. Assessment of neo-vascularization in HCC tissue was performed by staining $CD34^+$ cells in the resected samples obtained from patients with HCC. Immunohistochemical staining for CD34 was done as described in Materials and Methods. The $CD34^+$ cells were mainly confined to the cytoplasm of vascular endothelial cells as brownish yellow granules. Microvessels were represented by brownish yellow capillaries or small cell clusters. Representative results are shown. The panel shows a tumor cell area of a high grade.

B. The correlation is analyzed between TEM frequencies and the counts of $CD34^+$ cells (MVD values) in relevant patients. The panels show the correlation of peripheral (left: n=11) or intra-tumoral TEM frequencies (right: n=9) and MVD ($p = 0.0009$, $R^2 = 0.72$ and $p = 0.04$, $R^2 = 0.44$, respectively). Analysis was based on Pearson's correlation coefficient.

Fig. 5: Frequency of TEMs is superior as a diagnostic for HCC to common tumor markers or angiogenesis factor.

A. Correlation between TEM frequency and AFP (n=87) or PIVKA-II (n=81) was analyzed using Pearson's correlation coefficient. $P = 0.45$, $R^2 = 0.007$ or $P = 0.27$, $R^2 = 0.02$, respectively.

B. ROC analyses were performed in order to assess the diagnostic value of TEM frequency for differentiating HCC (n=89) from chronic liver disease (CLD, n=79) or liver cirrhosis (LC, n=30). The left panel shows the diagnostic value of TEMs, PIVKA-II, AFP and angiopoietin-2 (ANG-2) for HCC from CLD and the right panel shows those for HCC from LC, respectively.

Supplementary figure 1: Frequency of TEMs is comparable in HCC patients regardless of etiology.

The frequencies of peripheral TEMs are shown for HCC patients with or without HCV infection (n=89 and n=26, respectively). The group without HCV infection included patients with HBV

infection or those without HBV nor HCV (HBV-HCC and Non-B, Non-C [NBNC] HCC patients).

n.s. not significant by Mann-Whitney non-parametric U test.

Supplementary figure 2: Peripheral frequency of TEMs is not related to the overall survival in patients with HCC.

In patients with HCC who underwent RFA or the operation, the overall survival rate after the treatment was compared between those with TEM^{high} (frequency of TEMs $\geq$ 2.75; n=45) and TEM^{low} (frequency of TEMs < 2.75; n=44) using the Kaplan-Meier method, with the log-rank test for comparison. TEM^{high} and TEM^{low}, see Table 2. P=0.36

Supplementary figure 3: Correlation between the frequency of TEMs and serum levels of angiogenesis factors

All plots indicate the correlation between TEM frequency and VEGF, ANG-2, sVEGFR-1 or MIF. The numbers of patients examined were 32, 37, 23 and 31, respectively. Analyses were based on Pearson's correlation coefficient.

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Table.1: The clinical backgrounds of the subjects

Clinicopathologic characteristics	CH	LC	HCC
Gender: Male/female	21/28	13/17	63/26
Age: mean±SD	63.4±7.8	67.5±8.8	70.0±7.0
Alanine aminotransferase (IU/L)	56.1±43.5	54.0±28.6	46.3±29.5
Prothrombin time (%)	91.5±14.8	73.4±9.7	77.7±15.4
Platelet ($\times 10^4/mm^3$)	15.8±5.0	8.6±3.7	11.5±5.7
Albumin (g/dl)	4.0±0.3	3.5±0.4	3.5±0.5
Total bilirubin (mg/dl)	0.7±0.3	0.9±0.4	0.9±0.6
Child-Pugh grade: A/B	49/0	21/9	59/30
α-fetoprotein (ng/ml)	8.7±10.7	42.6±80.6	264.0±1281.7
TNM stage: I/II/III/IV			33/37/15/4
BCLC stage: A/B/C/D			44/13/26/6

TNM, Tumor, Lymph Node and Metastasis; BCLC, Barcelona-Clinic Liver Cancer; CH, chronic hepatisis; LC, liver cirrhosis; HCC, hepatocellular carcinoma

Table.2: The comparison of clinical parameters of HCC patients between those with higher frequency of TEMs and those with lower frequency

Clinicopathologic characteristics	Peripheral TEMs		
	High (n=45)	Low (n=44)	*P*
Gender: Male/female	32/13	31/13	1.000[†]
Age: mean±SD	71.4±6.6	68.7±7.3	0.065[‡]
Child-Pugh grade: A/B	24/20	34/10	**0.043**[†]
MELD score	9.1±2.3	8.2±1.8	**0.010**[§]
α-fetoprotein (ng/ml)	471.0±1785.2	52.3±101.4	0.101[§]
TNM stage: I+II/III+IV	35/10	35/9	1.000[†]
BCLC stage: A+B/C+D	25/20	32/12	0.123[†]
Vascular invasion: present/absent	4/41	2/42	0.677[†]
Tumor size: <3/>3cm	36/9	34/10	0.800[†]
Tumor number: single/multiple	21/24	25/19	0.399[†]
Alanine aminotransferase (IU/L)	51.5±34.5	41.0±22.6	0.251[§]
Prothrombin time (%)	73.3±14.5	82.6±15.0	**0.004**[§]
Platelet ($\times 10^4/mm^3$)	10.8±5.2	12.2±6.2	0.319[§]
Albumin (g/dl)	3.4±0.4	3.6±0.6	**0.045**[§]
Total bilirubin (mg/dl)	0.9±0.6	0.9±0.5	0.820[§]

P value of less than 0.05 was expressed by thick one.

[‡]Student's *t* test, [†]$\chi 2$ test or Fisher exact test, [§]Mann-Whitney *U* test.

TEMs, TIE2-expressing monocytes; MELD, model for endstage liver disease

Table.3: The assessment of diagnostic value of the TEMs, AFP and PIVKA-II by ROC analyses

Discrimination between	Parameter	AUC	Cut off value	Sensitivity (%)	Specificity (%)	Positive predictive values (%)	Negative predictive values (%)
HCC and CLD	TEMs	0.85	1.46	86.1	71	76.3	82.4
	AFP	0.69	10	65.9	43.9	65.9	56.1
	PIVKA-II	0.77	47	56.3	96.5	97.8	44.5
	ANG-2	0.62	2681	51.4	67.3	52.8	64
HCC and LC	TEMs	0.93	2.75	81.3	90	89.7	81.8
	AFP	0.61	10	71.9	32.1	54.8	50
	PIVKA-II	0.79	39	64.5	93.3	95.2	56
	ANG-2	0.57	2440	68.8	52	47.8	72.2

The optimal cut-off point was determined as those yielding the minimal value for $(1-\text{sensitivity})^2 + (1-\text{specificity})^2$. Such point with those sensitivity and specificity values is the closest to (0, 1) point on ROC curve. .

AUC, Area under the curve; TEMs, TIE2-expressing monocytes; CLD, chronic liver disease; LC, liver cirrhosis; ANG-2, angiopoietin-2

Supplementary Table.1: The inclusion and exclusion criteria for the enrollment of patients

- Eligible criteria for patients with hepatocellular carcinoma (HCC)

1) PS: 0～2 (ECOG PS)

2) Age: 40 - 80 years

3) Viable HCC was diagnosed by imaging analyses, such as CT and/or MRI.
Histological diagnosis is optional depending on the availability for tissue samples.

4) De novo as well as recurrent HCC are included

5) Patients who were tolerable for trans-catheter arterial chemo-embolization therapy, local ablation therapy and/or surgical resection of the tumor

- Eligible criteria for patients with chronic liver disease

1) PS: 0～2 (ECOG PS)

2) Age: 40 - 80 years

3) The presence of HCC was excluded by imaging analyses

4) Chronic hepatitis or liver cirrhosis was clinically diagnosed by biochemical and imaging examinations

- Exclusion criteria for patients

1) Pregnant women

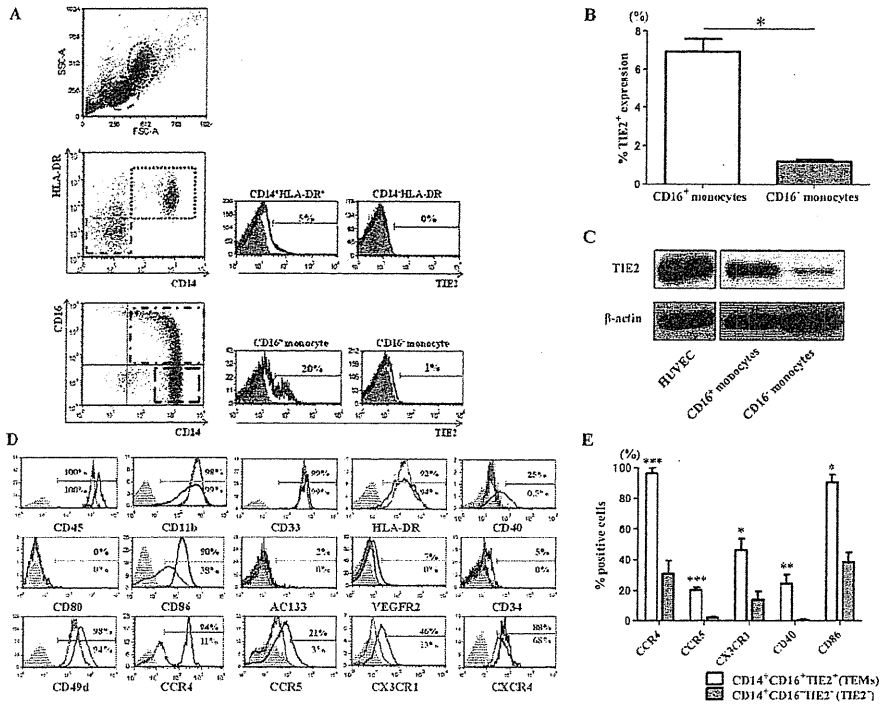
2) Patients with bleeding tendency (%prothrombin time<50%, platelet counts<30000/mm^3)

3) Patients with autoimmune hepatitis, primary biliary cirrhosis or primary sclerosing cholangititis

4) Patients with autoimmune diseases in any organs

5) Patients whom the attending doctors determined as ineligible

PS, Performance status; ECOG, Eastern Cooperative Oncology Group



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A. PBMC obtained from HCC patients were stained and analyzed by flow cytometry. CD14+ monocytes were divided into two distinct subsets, CD14++CD16- and CD14+CD16+ cells. These cells were examined for TIE2 expression. The numbers in the histograms depict the percentages of gated cells. Representative plots from three patients are shown.
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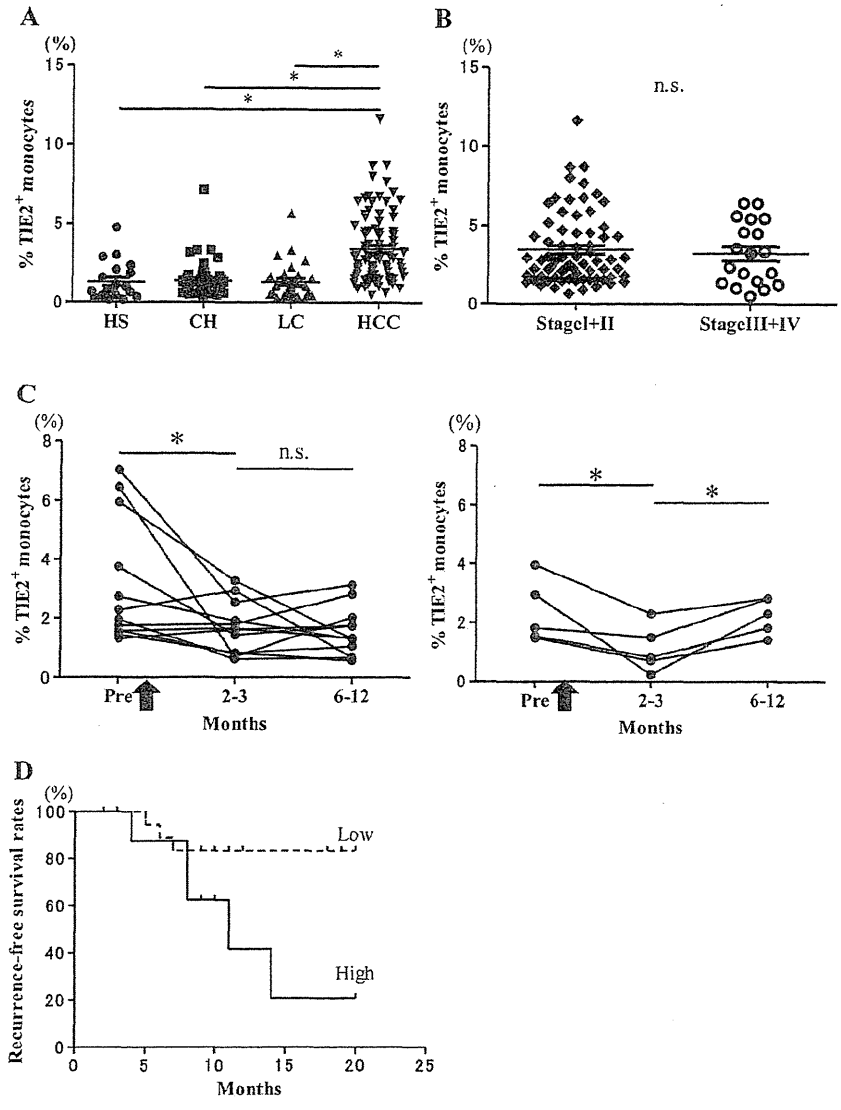

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C. In patients who underwent RFA therapy or resection of HCC, the frequencies of TEMs in CD14+ monocytes were examined serially after confirmation of complete ablation or operation to remove HCC lesions. The bold arrows depict the time point of the RFA therapy or the operation. Both panels depict the frequencies of TEMs in patients before and after the treatment. The left panel shows the results of TEMs in patients without HCC recurrence (n=12) as assessed by CT/MRI examinations, while the right panel shows the results in patients with HCC recurrence (n=5), respectively. *: $p<0.05$ by Paired t-test.

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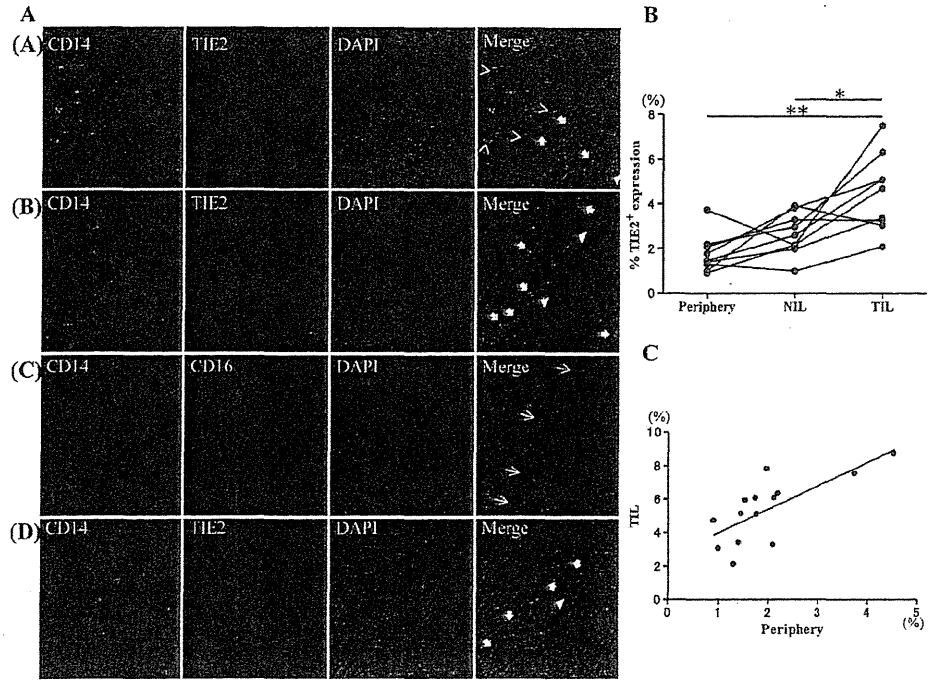

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A. Immunofluorescence staining was performed as described in Materials and Methods. The staining for CD14 (green), CD16 (red) and TIE2 (red) identifies CD14+TIE2-, CD14+TIE2+, CD14+CD16- cells and CD14+CD16+ monocytes in human liver tissue (blue: nuclei counterstained with Dapi). Representative results of the resected samples obtained from 12 HCC patients are shown. The panels show CD14+TIE2+ cells (A, B, D) and CD14+CD16+ cells (C) in the perivascular area of HCC tissue (magnification, 400x), Bold arrows depict CD14+TIE2+ cells, thin arrows CD14+CD16+ cells, bold arrowheads vascular endothelial cells (TIE2+CD14-) and thin arrowheads CD14+TIE2- cells.

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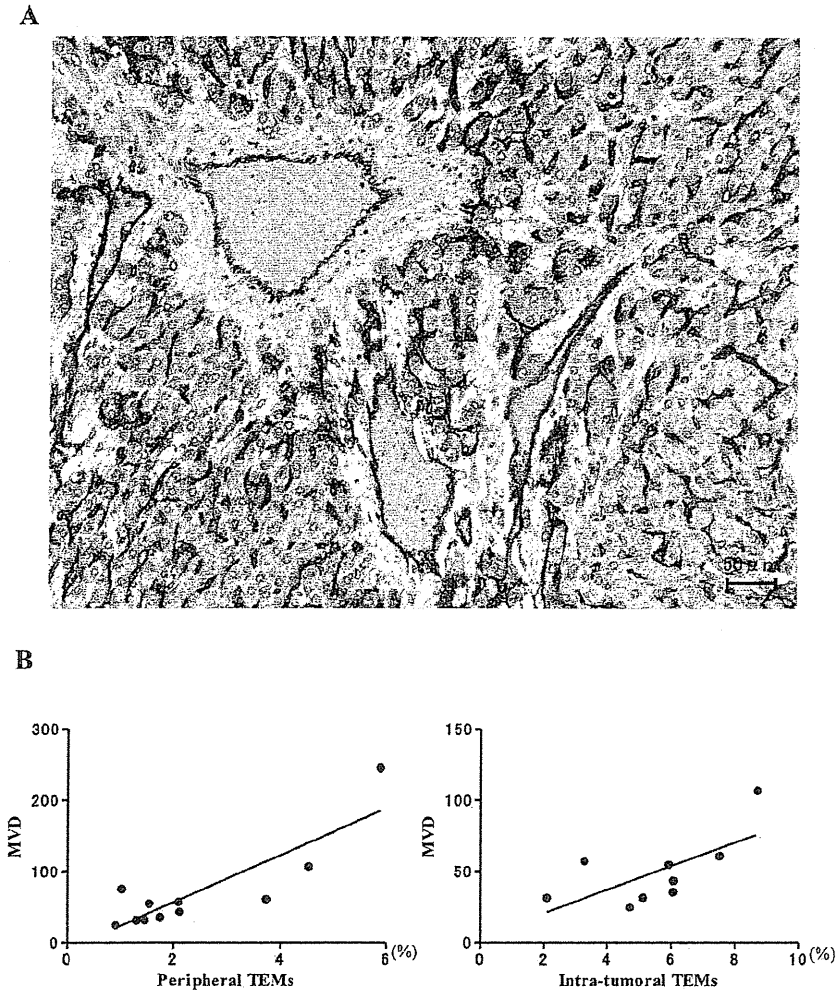

Fig. 4: The degree of microvessels in HCC is correlated with the frequency of TEMs
A. Assessment of neo-vascularization in HCC tissue was performed by staining CD34+ cells in the resected samples obtained from patients with HCC. Immunohistochemical staining for CD34 was done as described in Materials and Methods. The CD34+ cells were mainly confined to the cytoplasm of vascular endothelial cells as brownish yellow granules. Microvessels were represented by brownish yellow capillaries or small cell clusters. Representative results are shown. The panel shows a tumor cell area of a high grade.
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