Expression of vascular endothelial growth factor receptors is closely related to the histological grade of hepatocellular carcinoma

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Abstract. Angiogenesis is important for tumor growth, and is regulated by angiogenetic factors such as vascular endothelial growth factor (VEGF). In the present study, we investigated whether or not expression of VEGF receptors (VEGFRs) is related to the proliferation of tumor cells in hepatocellular carcinoma (HCC). We simultaneously stained proliferation marker Ki-67 antigen and either VEGFR1 (Flt-1) or VEGFR2 (Flk-1) on paraffin-embedded tissue sections from 50 cases of surgically resected human HCC. Based on the staining pattern of VEGFRs, we classified the cases into 4 categories; receptor double-negative, Flt-1 single-positive, Flk-1 single-positive, receptor double-positive. Interestingly, the Ki-67 index was significantly lower in receptor double-negative cases in comparison to that in either Flt-1 single-positive or Flk-1 single-positive cases (P=0.0491, P=0.0196, respectively). Moreover, the index was also significantly lower in receptor double-positive cases in comparison to either Flt-1 single-positive or Flk-1 single-positive cases (P=0.0026, P<0.0001, respectively). We further investigated 35 cases showing a Ki67 index >10% to determine the expression of VEGFRs on Ki-67 antigen-positive proliferating cells. Surprisingly, the histological grade of HCC and the expression pattern of VEGFRs showed a characteristic relation; the well-differentiated HCC cases were all distributed in the Flk-1-positive group (7/7), moderately differentiated HCC cases were distributed in either the Flt-1 or Flk-1 single-positive group (20/21), and poorly differentiated HCC cases were predominantly distributed in either the receptor double-negative or double-positive group (6/7). These findings suggest that the expression pattern of VEGFRs influences the histological differentiation of HCC.

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Introduction

Angiogenesis is crucial for the growth of solid tumor (1,2). Vascular endothelial growth factor (VEGF) is well known as one of the most important factors involved in angiogenesis (3,4). It has been reported that many malignant tumor cells produce VEGF and that the expression level of VEGF is well correlated with tumor growth, metastasis, and poor prognosis (5-7). Recently, anti-angiogenetic therapy utilizing inhibitors of VEGF or VEGF receptors (VEGFRs) has been enthusiastically adapted to cancer therapy and resulted in a better prognosis in combination with chemotherapeutic agents (8,9). In this therapy, tumor endothelium was targeted in an attempt to block binding of VEGF to VEGFR, which resulted in slow tumor growth via inhibition of endothelial cell growth. Boocock *et al* first reported that VEGFR was expressed on ovarian carcinoma tissue sections and suggested that VEGF contributed to tumor growth via an autocrine mechanism (10). Recent reports support that VEGFR expressed on tumor cells and VEGF played a role in proliferation of tumor cell growth via an autocrine mechanism (11-13). They suggested that anti-angiogenetic therapy utilizing inhibitors of VEGF and VEGFRs was directly effective in inhibiting tumor growth, in addition to growth inhibition through inhibition of endothelial cell growth (13).

VEGF induces proliferation, migration and survival of endothelial cells through interaction with its receptors; VEGF receptor 1 (VEGFR1/Flt-1) and VEGF receptor 2 (VEGFR2/Flk-1) (3). VEGFR2 is reported to induce vascular proliferation, migration, and survival (14), whereas VEGFR1 inhibits the role of VEGFR2 (15). In contrast, Yoshiji *et al* recently reported that blocking of VEGFR1 alone inhibited tumor growth and that blocking of VEGFR1 with anti-VEGFR2 antibody synergistically inhibited tumor growth in an animal model (16). This report suggested that VEGFR1 might induce

Table I. Clinicopathological characteristics of 50 surgically resected HCCs used in the present study.

Factors		Number of patients
Age	<60 years	13
	≥60 years	37
Sex	Male	38
	Female	12
Virus markers	HBs Ag (+)	9
	HCV Ab (+)	40
	HBs Ag (-) and HCV Ab (-)	1
Liver cirrhosis	Present	34
	Absent	16
Tumor size	≤40 mm	32
	>40 mm	18
Grade of differentiation	Well	10
	Moderately	33
	Poorly	7
Capsule formation	Present	32
	Absent	18
Septum formation	Present	43
	Absent	7
Portal vein invasion	Present	10
	Absent	40
Intrahepatic metastasis	Present	21
	Absent	29

tumor proliferation by itself. Therefore, further elucidation of whether or not VEGFR1 contributes to tumor growth is warranted.

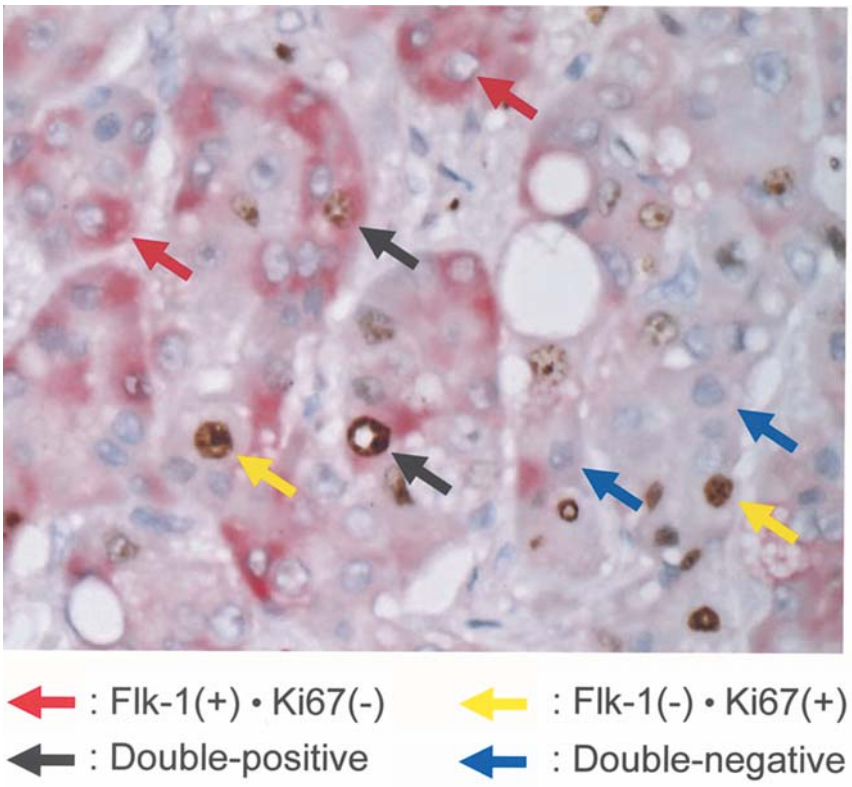
There is no report that investigates the relationship between VEGFR expression on the HCC cell and proliferation of HCC. Therefore, the aim of this study is to clarify whether or not VEGFR expression correlates with tumor cell proliferation *in vivo* using tissue sections from hepatocellular carcinoma patients. We simultaneously observed VEGFR1 or VEGFR2 expression in combination with cellular proliferation marker Ki67 expression on tissue sections from hepatocellular carcinoma patients.

Materials and methods

Patients. Tissue samples from fifty cases of surgically resected hepatocellular carcinoma (HCC) (between January 1998 and December 2003) at Gifu University hospital were analyzed in this study. These cases were numbered chronologically. There were 38 males and 12 females, and the average patient age was 66.4 years (range, 41 to 85 years.). Patients with a history of hepatectomy or preoperative chemotherapy were excluded from the analysis. Clinicopathological factors of analyzed patients were classified according to the criteria of the Japanese Research Council on Hepatocellular Carcinoma and summerized in Table I. The analyses were performed according to the guidelines of the ethics committee of Gifu University School of Medicine.

Immunohistochemical analyses. Surgically resected samples were fixed in 10% paraformaldehyde in phosphate buffered saline (PBS) for 24 h, then paraffin-embedded tissue blocks were prepared. Three μm-thick sections were prepared and submitted for standard dewaxing and hydration processes. Sections were immersed into 10 mM citrate buffer, pH 7.0, then autoclaved for 20 min at 121˚C. After washing with double distilled water (D.D.W.), the section was dried using a fan, and the outline of the section was marked using a PAP pen (Dako Cytomations Japan, Kyoto). After an additional 30-min drying process, the section was rinsed with 0.3% skim milk in 50 mM Tris-HCl, pH 7.6 (Tris buffer), then rinsed with Tris buffer to remove excess skim milk from the section. After shaking off the Tris buffer, 1-50x diluted anti-Flk-1 mouse monoclonal antibody (sc-6251, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or diluted anti-Flt-1 rabbit polyclonal antibody (Spring Bioscience, Fremont, CA), was applied to the section at room temperature (R/T) and incubated for 1 h in a humidified chamber. After washing with Tris buffer for 3x 10 min, either alkaline phosphatase conjugated goat anti-mouse IgG, Histofine Simple Stain AP (M) (Nichirei, Tokyo, Japan), or alkaline phosphatase conjugated sheep anti-rabbit IgG, Histofine Simple Stain AP (R) (Nichirei) was applied for 1 h at R/T. After washing with Tris buffer for 3x 10 min, the reaction was visualized with Dako New Fuchsin substrate system (Dako Cytomations Japan). To terminate the reaction, the section was submerged into 4% paraformaldehyde in Tris

A



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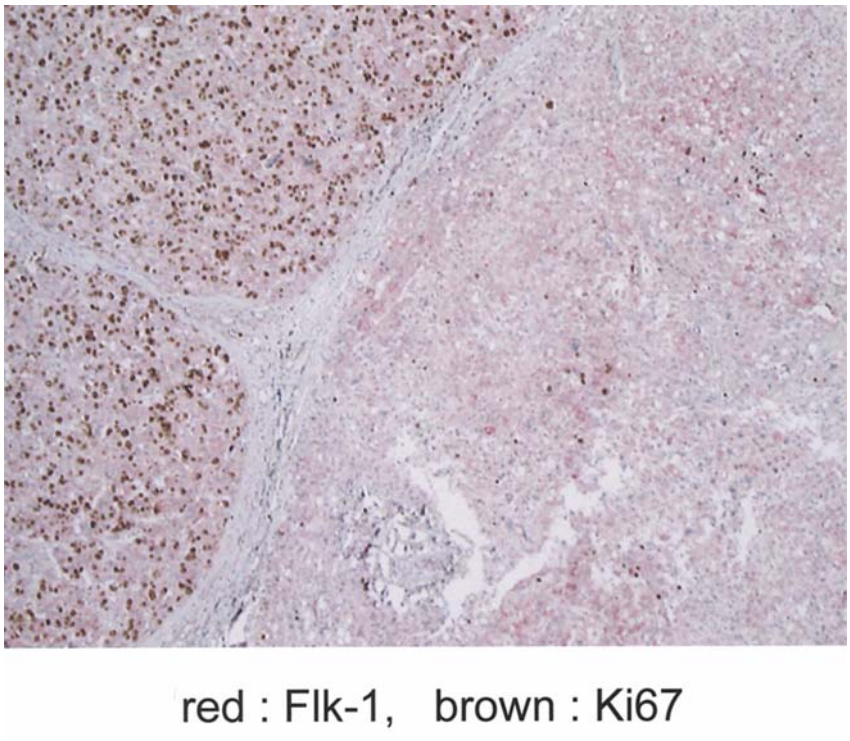

Figure 1. Immunohistochemical double staining of VEGFR2 and Ki67. (A) Case 44 is shown as a representative staining pattern. Intra-cytoplasmic staining of Flk-1 is observed as red, and nuclear staining of Ki-67 antigen is observed as brown. The red arrows indicate Flk-1(+)-Ki67(-) cells, yellow arrows indicate Flk-1(-)-Ki67(+) cells, black arrows indicate double-positive cells, and blue arrows indicate double-negative cells. (B) In this case (case 31), the Ki67 index obviously differed at various sites within the nodule. Therefore, we analyzed areas of the nodule independently.

buffer for 10 min and washed with Tris buffer for 3x 10 min and stored at 4˚C in the dark until the next step was performed.

For Ki67 staining, an Flt-1 or Flk-1 stained section was submerged into 10 mM citrate buffer, pH 7.0, then autoclaved at 121˚C for 20 min to degenerate the antibody previously reacted on the section. After rinsing with D.D.W., the specimen was dried and submerged in 0.3% hydrogen peroxide in Tris buffer for 20 min at R/T to block endogeneous peroxidase activity.

After washing with Tris buffer for 3x 10 min, the section was rinsed with 0.3% skim milk in Tris buffer, then rinsed with Tris buffer to remove excess skim milk from the section.

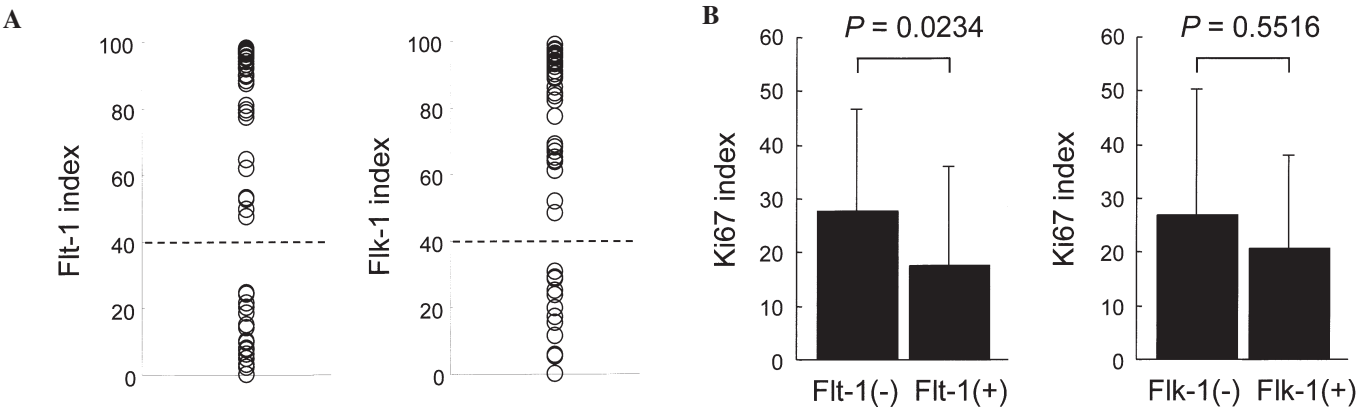

Figure 2. Analyses of VEGFR distribution and Ki67 index on HCC specimens. (A) Distribution of Flt-1 index and Flk-1 index in HCC specimens. Specimens showing <40% in either index were termed either Flt-1-negative or Flk-1-negative, the remaining specimens were termed either Flt-1-positive or Flk-1-positive. (B) Ki67 index was compared with Flt-1- or Flk-1-negative group versus the positive group.

After shaking off the Tris buffer, 1-50x diluted anti-Ki67 mouse monoclonal antibody (clone MIB-1, Dako Cytomations Japan) was applied to the section and incubated for 1 h at R/T. After washing with Tris buffer for 3x 10 min, horse radish peroxidase conjugated goat anti-mouse IgG, Histofine Simple Stain PO (M) (Nichirei) was applied for 1 h at R/T. After washing with Tris buffer for 3x 10 min, the reaction was visualized with 0.03% 3,3'-diaminobenzidine, tetrahydrochloride, 0.3% hydrogen peroxide in Tris buffer. After fixing with 4% paraformaldehyde in Tris buffer for 10 min, regular nuclear staining with hematoxylin and a dehydration process were performed and the section was submitted for observation.

Assesment of the stained sections. To assess specimens, each sample was observed at magnification of x400, and all tumor cells observed in 5 fields were counted. All tumor cells were classified into 4 classes according to the staining for Flt-1, Flk-1, and Ki67; Flt-1$^+$Ki67$^+$, Flt-1$^+$Ki67$^-$, Flt-1$^-$Ki67$^+$, and Flt-1$^-$Ki67$^-$ cells for Flt-1 and Ki67 double-stained specimens, and Flk-1$^+$Ki67$^+$, Flk-1$^+$Ki67$^-$, Flk-1$^-$Ki67$^+$, and Flk-1$^-$Ki67$^-$ cells for Flk-1 and Ki67 double-stained specimens respectively. Flt-1 or Flk-1 index was defined as Flt-1- or Flk-1-positive cells versus total tumor cells. Ki67 index was defined as Ki67-positive cells versus total tumor cells.

Statistical analyses. Mann-Whitney's U-test was performed and a $p<0.05$ was considered significant.

Results

Ki67 index was significantly lower in Flt-1-positive cases. Fig. 1A shows the staining pattern for a Flk-1 and Ki67 double-stained HCC section (case 44). Among the 50 cases, there were tumor nodules with different staining patterns for either Ki67, Flt-1 or Flk-1 despite confirmation of non-necrotic area. In these cases, we analyzed each nodule independently, 55 nodules in total (Fig. 1B).

Fig. 2A shows the Flt-1 or Flk-1 index in all cases. The Flt-1 index was distributed from 0 to 98% (average, 47.7±40.4%), and Flk-1 index was distributed from 0 to 99% (average, 64.5±34.7%). There appeared to be gaps at 40% in both the Flt-1 and Flk-1 indices. Therefore, we sub-classified the groups showing >40% on the Flt-1 index as Flt-1-positive and <40% on the Flt-1 index as Flt-1-negative, >40 % on the Flk-1 index as Flk-1-positive, and <40% on the Flk-1 index as Flk-1-negative group. Based on this sub-classification, the percentage in the Flt-1-positive group was 52.7% (29 of 55 specimens), and the percentage in the Flk-1-positive group was 72.7% (40 of 55 specimens) (Fig. 2A). Ki67 index in all cases was distributed from 2 to 85% (average, 23.2±20.6%).

Fig. 2B shows that the distribution of Ki67 index among each sub-class was 27.7±19.1% in Flt-1-negative cases, 17.6±18.3% in Flt-1-positive cases, 26.8±23.5% in Flk-1-negative cases, and 20.7±17.3% in Flk-1-positive cases. There was a significant difference in the Ki67 index between the Flt-1-negative group and Flt-1-positive group (P=0.0234).

Ki67 index was significantly low in the Flt-1 and Flk-1 double-positive cases based on sub-classification of VEGF receptor expression pattern. We aligned the sections in sequence and analyzed Flt-1 and Flk-1 distribution patterns in the same tumor nodules. Then we analyzed the Ki67 index of the same tumor nodules in relation to the Flt-1 and Flk-1 findings (Fig. 3A). As shown in Fig. 3B, the Ki67 index in specimens that were receptor double-negative, Flt-1 single-positive, Flk-1 single-positive, and receptor double-positive was 17.3±21.0%, 37.6±22.9%, 32.3±16.8%, 11.2±10.9%, respectively. There was a significantly lower Ki67 index in receptor double-negative cases in comparison to that in either Flt-1 or Flk-1 single-positive case (P=0.0491, P=0.0196, respectively). Interestingly, the Ki67 index in receptor double-positive cases was also significantly lower in comparison to either Flt-1 or Flk-1 single-positive groups (P=0.0026, P<0.0001, respectively) (Fig. 3B).

Fig. 3C shows a representative case in which the Ki67 index was higher in the Flt-1 single-positive area in comparison to that in the receptor double-negative area. Fig. 3D shows a representative case in which the Ki67 index was higher in an Flk-1 single-positive area in comparison to that in the receptor double-positive area.

Histological grade of HCC was well related to VEGFR expression pattern in proliferative phase HCC cells. Because we stained the VEGF receptor and Ki67 simultaneously on

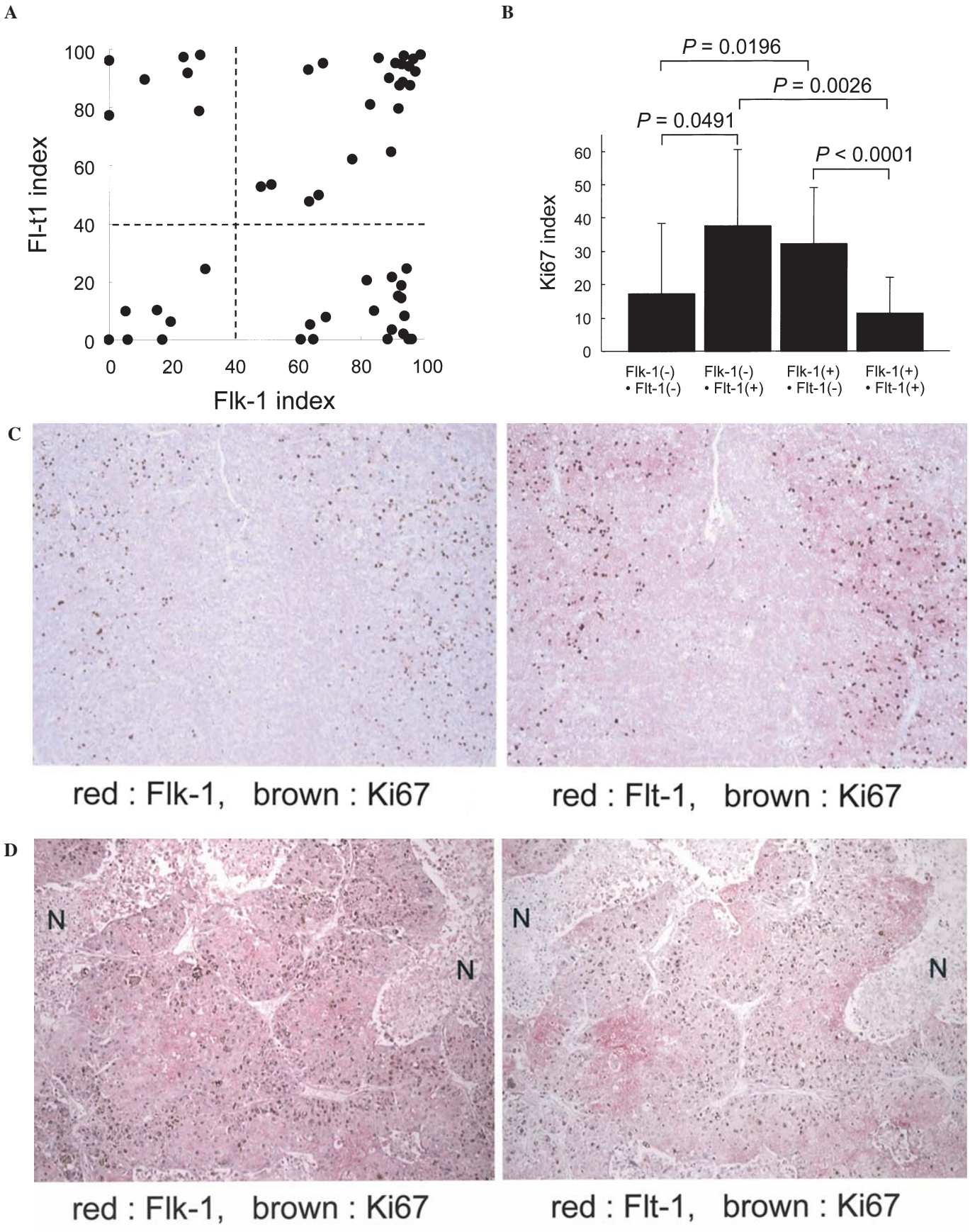

Figure 3. Analyses of Ki67 index based on VEGF receptor expression on HCC specimens. (A) Each specimen was classified based on the Flt-1 and Flk-1 indices. The cut-off between each group was less than or more than 40% for each index. (B) Ki67 index was compared in each group. (C) A representative case showing both an Flk-1(-)-Flt-1(+) area and an Flk-1(-)-Flt-1(-) area in the same specimen (case 50). The former area showed a high Ki67 index and the latter area showed a low Ki67 index. (D) A representative case showing both an Flk-1(+)-Flt-1(+) area and an Flk-1(+)-Flt-1(-) area in the same specimen (case 17). The former area showed a low Ki67 index and the latter area showed a high Ki67 index. N indicates a necrotic area, which was excluded from analysis.

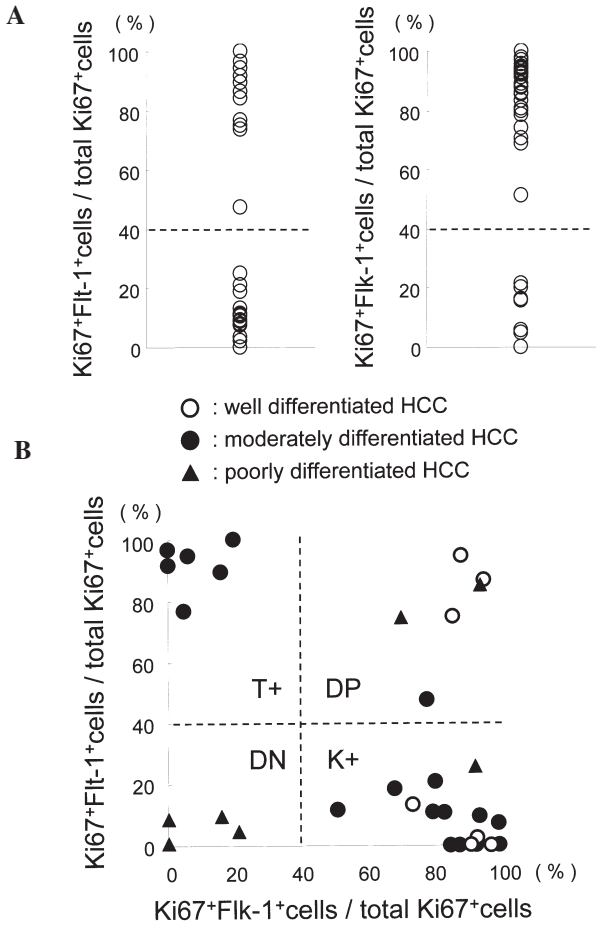

Figure 4. VEGF receptor distributions in Ki67-positive cells and relation with histological differentiation. (A) Ki67-positive in each specimen were classified based on the Flt-1 and Flk-1 indices. The cut-off between each group was less than or more than 40% of each index. (B) Combined distribution of Flt-1 and Flk-1 index in Ki67-positive cells. DN; Flt-1 and Flk-1 double-negative group, T+; Flt-1(+)-Flk-1(-) group, K+; Flt-1(-)-Flk-1(+) group, and DP; Flt-1 and Flk-1 double-positive group. In terms of histological differentiation, open circle indicate well-differentiated HCC, filled circles indicate moderately differentiated HCC, and filled triangles indicate poorly differentiated HCC.

the same section, we tried to evaluate Ki67-positive cells for Flt-1 or Flk-1 index. For this purpose, we selected 35 cases in which the total Ki67 index of both Flk-1 double-stained and Flt-1 double-stained sections was >10%. As shown in Fig. 4A, the Flt-1 index was distributed from 0 to 100% (average, 34.0%) in Ki67-positive cells. While, the Flk-1 index was distributed from 0 to 100% (average, 63.5%) in Ki67-positive cells. When we selected 40% as the cut-off for Flt-1- or Flk-1-positive groups, the Flt-1-positive cases comprised 37.1% (13/35), while Flk-1-positive cases comprised 71.4% (25/35), respectively.

On the other hand, both receptor double-negative cases comprised 11.4% (4/35), Flt-1-single-positive cases comprised 17.1% (6/35), Flk-1-single-positive cases comprised 54.3% (16/35), and both receptor-positive cases comprised 17.1% (6/35), respectively. That is, Ki67-positive cells tended to show an Flk-1 single-positive phenotype (Fig. 4B). In addition, based on the histological differentiation analysis, moderately differentiated hepatocellular carcinoma tended to show Flt-1 single-positive or Flk-1 single-positive phenotypes (100 and 73.7% respectively), while all 4 receptor double-negative cases showed poorly differentiated hepatocellular carcinoma (Fig. 4B).

Discussion

VEGF plays a role in the induction of proliferation of endothelial cells through its receptors (3). VEGF expression was initially identified in endothelial cells, and was considered specific in endothelial cells (17-19). However, VEGF was later reported to be expressed on monocytes (20), melanoma, bladder cancer and breast tumor cells (11-13), and autocrine process of VEGF and its receptors is important to maintain proliferation of tumor cells (11-13). Although Yamaguchi *et al* reported that hepatocellular carcinoma cells did not express either Flt-1 or Flk-1 (21), VEGF receptor expression was reported in head and neck cancer, prostate cancer, and gastric cancer tissue (22-24). Although there are many VEGF receptors including VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), VEGFR-3 (Flt-3), and Neurophilin-1 (NP-1), there have been many reports related to the functions of VEGFR-1 and VEGFR-2 (25-31). In the present study, we examined Flt-1 and Flk-1 expression on HCC tissue sections and showed the expression of Flt-1 and Flk-1 on the paraffin-embedded tissue sections. Fig. 3B shows both Flt-1 single-positive cases and Flk-1 single-positive cases to have a higher Ki67 index in comparison to that on receptor double-negative cases. In addition, Ki67 index was significantly lower in both receptor double-positive cases in comparison to that in single-positive cases. These data suggest that either Flt-1 or Flk-1 receptor single-positive phenotype would be important for carcinoma cell growth because the receptor double-negative phenotype showed a low Ki67 index. In addition, our data also suggested that the expression of either receptor on the tumor cells would interfere with the cell growth induced by the other.

To clarify our findings, we must discuss the present understanding of Flk-1 and Flt-1. Shalby *et al* suggested that Flk-1 played a role in the proliferation of endothelial cells utilizing Flk-1 null mice (32). Takahashi *et al* showed that Flk-1 activation through VEGF resulted in proliferation of endothelial cells (14). Moreover, Wu *et al* also reported the Flk-1 mediated proliferation of bladder tumor cells (13).

However, the role of Flt-1 in cell proliferation remains contradictory. Fong *et al* suggested that Flt-1 played a role in angiogenesis but did not in proliferation of endothelial cells utilizing Flt-1 null mice (33). Park *et al* showed that Flt-1 itself was not directly related to proliferation, but it did participate in cell growth inhibition of Flk-1-mediated cell-proliferation as a decoy receptor (34). Zeng *et al* showed that Flt-1 inhibits Flk-1-mediated proliferation signals (35). Landgren *et al* showed that Flt-1 could induce cell proliferation in combination with placental growth factor (PLGF), the expression of which is restricted to the placenta (36). Fan *et al* showed that Flt-1 expression in colon cancer cells was not related to proliferation but rather to migration, invasion or colony formation (37).

Therefore, the above reports support our findings that Flk-1 single-positive cases had a high Ki67 index, while Flt-1

and Flk-1 double-positive cases had a low Ki67 index. Based on finding that Flt-1 single-positive cases had a high Ki67 index, Flt-1 may be related to cell proliferation in hepatocellular carcinoma. However, the mechanism of how Flt-1 single expression would contribute to inducing the $Ki67^+$ proliferation phase in tumor cells is not obvious. Regarding this point, Vincent *et al* reported that Flt-1 played a role in cellular proliferation in primary multiple myeloma cells that express Flt-1 but Flk-1 does not (38). Thus, our findings, and collectively suggest that Flt-1 induces proliferation signals only when Flt-1 is expressed alone.

Ki67 can label the cell in the proliferative phase (S, G2, M, and G1), and indicates cell proliferative activity (39). In this study, we evaluated the relationship between tumor cell proliferative activity and Flt-1 or Flk-1 expression in formalin-fixed paraffin-embedded tissue sections because the localization of Ki67 antigen and VEGFR (Flt-1 or Flk-1) differ, with the former being located in the nucleus and the latter in the cytoplasm. That is, our double-staining procedure would be useful to evaluate the relationship between proliferation activity and another factor that is not located to the nucleus.

Xie *et al* performed Ki67 and VEGF double-staining for breast cancer tissue implanted in rats, and showed the correlation of VEGF expression and Ki67-staining (40). Although they examined the correlation of Ki67 expression and Flk-1 or Flt-1 expression, they did not examine many different cases. Regarding this point, our double-staining methods for human paraffin-embedded tissue sections were very useful, providing new knowledge that not only Flk-1 but also Flt-1 might participate in proliferation of hepatocellular carcinoma cell growth, when Flt-1 is expressed without Flk-1 expression.

The discussion cited above speculated on whether VEGFR expression is related to the Ki67 index. Because we stained Ki67 antigen and VEGFR simultaneously on the same sections, we were able to demonstrate that Ki67-positive cells showed characteristic patterns of VEGFR expressions. Interestingly, this analysis showed that there was a relation between the VEGFR expression patterns and the degree of histological differentiation (Fig. 4C). That is, moderately differentiated HCC cells in the proliferative phase tended to express either Flt-1 or Flk-1 alone. However, poorly differentiated HCC cells in the proliferative phase tended to show Flt-1 and Flk-1 double-negative or double-positive expression pattern. Moreover, well differentiated HCC cells in the proliferative phase tended to show the Flk-1- positive phenotype, regardless of the Flt-1 expression. Indeed, Ferrer *et al* observed a relationship between histological grade and Flk-1 expression in human prostate cancer tissue sections (22).

Several reports suggested that VEGF expression was related to hepatocarcinogenesis from the early stages (41,42). In addition, VEGF expression is increased in aberrant expression of tumor suppressor p53, which is frequently shown in moderately or poorly differentiated HCC but not in well-differentiated HCC (43,44). Therefore, the present findings were very interesting because the VEGFR expression pattern in proliferating carcinoma cells correlated well with the histological grade.

In conclusion, in the present study, we showed the relationship between histological grade of HCC and expression pattern of VEGFRs in addition to showing the relationship between VEGFR expression and Ki67 index. This is the first report to show a relationship between histological grade of HCC and VEGFR expression pattern in relation to the proliferative activity of tumor cells by staining Ki67 and VEGFR simultaneously in the same sections.

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