Correlation of EPHA2 overexpression with high microvessel count in human primary colorectal cancer

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Evidence suggests that the erythropoietin-producing hepatocellular (EPH) receptor tyrosine kinases (RTKs) and their ephrin (EFN) ligands are involved in human carcinogenesis. Expression of two of them, EFNA1 ligand and its receptor, EPHA2, has been proposed to contribute to tumor-induced neovascularization. Colorectal cancers were examined for expressions of EPHA2 and its ligand EFNA1 by semi-quantitative RT-PCR, and double-immunostained for EPHA2 and CD34. Microvessels in the tumors were counted. Double-staining was also performed in 25 cases of adenoma with focal cancer for comparison. Trends of overexpression of both EPHA2 and EFNA1 was found in tumor tissue compared to the corresponding normal tissue in the same specimen [22/37 (59.5%) and 25/37 (67.5%), respectively; *P*=0.100 for EPHA2 and *P*=0.009 for EFNA1]. Overexpression of EPHA2 and EFNA1 was noted more frequently in the early stage than in the late stage [EPHA2, 15/21 (71.4%) vs. 7/16 (43.8%), *P*=0.007; EFNA1, 15/21 (71.4%) vs. 10/16 (62.5%), *P*=0.007]. Both EPHA2 and EFNA1 were more frequently overexpressed in smaller tumors (less than 5 cm) than in larger tumors [EPHA2, 15/21 (71.4%) vs. 7/16 (43.8%), *P*=0.017; EFNA1, 16/21 (76.2%) vs. 8/16 (50%), *P*=0.001]. Tumors less than 5 cm in diameter and in stages I and II were significantly more likely to overexpress EPHA2 and EFNA1 (*P*=0.001 for EPHA2, *P*=0.001 for EFNA1). Microvessel counts (MVCs) after immunostaining for CD34 were significantly correlated (*r*=0.343, *P*=0.037) with overexpression of EPHA2. EPHA2-expressing focal cancer also surrounded microvessels in adenomas with focal cancers. These findings suggest an involvement of EPHA2 in colon carcinogenesis, mainly in stages I and II, and probably through their effect on microvessel induction. (Cancer Sci 2004; 95: 136–141)

Receptor tyrosine kinases (RTKs), which are often the products of transforming oncogenes, have been widely implicated in the generation and progression of common human tumors.[1)] The erythropoietin-producing hepatocellular (EPH) receptors represent the largest known family of RTKs and they are activated by interaction with cell-surface ligands, termed ephrins (EFNs). EPH receptors have been classified into two subfamilies, EPHA and EPHB, according to their preference for either glycosylphosphatidylinositol (GPI)-anchored EFNA ligands or transmembrane EFNB ligands, and 14 receptors and eight ligands have been identified in vertebrates thus far. The EPH family receptor kinases and their EFN ligands are well known to be involved in fundamental developmental processes of the nervous system, including axon guidance,[2)] axon fasciculation,[3)] neural crest cell migration,[4)] acquisition of brain subregional identity,[5)] and neuronal cell survival.[6)] Evidence has suggested that one member of the EPH family, EPHA2, and one of its ligands EFNA1, are associated with tumorigenesis and tumor neovascularization.[7,8)] For example, forced overexpression of EPHA2 causes tumorigenesis by mammary epithelial cells *in vitro*, and its ligand, EFNA1, has been found to involved in TNF-α-induced angiogenesis.[7,9)] A recent paper reported that EFNA1 ligand and its receptor, EPHA2, are expressed by human breast cancer cells and human Kaposi's sarcoma cells during tumor neovascularization.[10)] However, expression of EPH receptors and EFNs in gastrointestinal tract tumors has seldom been documented.

In this study, we investigated the expression of EPHA2 and its ligand, EFNA1, in colorectal cancer specimens by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis and we counted CD34-positive microvessels surrounding the tumors to identify the relation between colorectal cancer and the EPHA2/EFNA1 system in terms of microvessel induction.

Materials and Methods

Tissues. RT-PCR samples of human colorectal cancer specimens and corresponding normal tissue were obtained from surgical resections performed at the Hamamatsu University School of Medicine between 1999 and 2002. The clinicopathological characteristics of 37 colorectal cancer patients from whom the specimens were obtained. are shown in Table 1. Histologically, the specimens consisted of 33 differentiated adenocarcinomas, and four non-differentiated adenocarcinomas (one poorly differentiated adenocarcinoma and three mucinous-type colorectal adenocarcinoma). There were two early adenocarcinomas and 35 advanced adenocarcinomas. Pathological TNM (tumor, node, and metastasis) classification yielded 21 stage I and II cases, and 16 stage III and IV cases. All of the specimens were immediately frozen in liquid nitrogen and stored at −80°C until RNA preparation. This project had been approved by the Institutional Review Board (IRB) of Hamamatsu University School of Medicine (12-11, 12-12 (IRB numbers of HUSM)).

RNA extraction and reverse transcription (RT). Total cellular RNA was extracted by using the RNA extraction reagent ISOGEN (Nippon Gene, Tokyo) according to the manufacturer's protocol. Single-stranded cDNA was prepared from total RNA and 1 μg of oligo dT primer (Life Technologies, Rockville, MD) in a total volume of 20 μl containing Moloney murine leukemia virus reverse transcriptase (Life Technologies) and RNase inhibitor (Toyobo, Tokyo).

Semi-quantitative RT-PCR analysis. The RT-PCR method used in this study has been described previously.[11)] To quantify human EPHA2, EFNA1, and β-actin mRNA by RT-PCR, the corresponding cDNA was diluted in water, and mixed in a final volume of 20 μl with 0.625 μmol/liter of primer pairs, 1 U of *Taq* DNA polymerase, and 1 μCi of ^{32}P-dCTP. Amplification was performed in a DNA thermal cycler (PC-700, ASTEC, Fukuoka). For the human β-actin control, 30 cycles of denaturation were performed for 45 s at 94°C, followed by primer annealing for 1 min at 59°C, polymerization for 1 min at 72°C, and final

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Table 1. Patients' profile

Total number		37
male		22
female		15
Median age (range)		66 (46–86)
Location	colon	25
	rectum	12
Pathology	differentiated	33
	poorly differentiated	1
	mucinous	3
Clinical status	early	2
	advanced	35
Clinical stage	I, II	21
	III, IV	16
Tumor size	5 cm>	21
	5 cm<	16
Distant metastases	liver	2
	lung	1

extension for 10 min at 72°C. For EPHA2 and EFNA1, 35 cycles of denaturation were performed for 45 s at 94°C, primer annealing for 1 min at 59°C, and polymerization for 1 min at 72°C, and final extension was performed for 10 min at 72°C. Under these conditions, the PCR products were collected during the exponential phase of amplification for β-actin, EPHA2, and EFNA1. A negative control tube was always added to the assay to exclude the possibility of DNA contamination of each of the reactions. The integrity of the RNA obtained from the clinical samples was confirmed by demonstrating the presence of β-actin mRNA in the same samples. The size of the β-actin, EPHA2, and EFNA1 was 121 base pairs (bp), 260 bp, and 230 bp, respectively. The primers were: (a) EPHA2, 5′-GCAACATCCTCGTCAACAGC-3′ (sense primer) and 5′-TGGCTTTCATCACCTCGTGG-3′ (antisense primer); (b) EFNA1, 5′-AACAAGCTGTGCAGGCATGG-3′ (sense primer) and 5′-CTCCACAGATGAGGTCTTGC-3′ (antisense primer); and (c) β-actin, 5′-GCTACGTCGCCCTGGACTTC-3′ (sense primer) and 5′-AGCGGAACCGCTCATTGCCA-3′ (antisense primer). The PCR products were separated by electrophoresis on 6% polyacrylamide gels, which were then dried and subjected to autoradiography and image analysis (MacBAS, Fuji Film, Tokyo). The band density values, including that of β-actin, were transformed logarithmically, and the count of each RNA message was calculated as a ratio to the count of β-actin.

Double-immunostaining of EPHA2 and CD34. Antibody used to detect EPHA2 was purchased from Santa Cruz (Santa Cruz, CA), and antibody to detect CD34 was purchased from DAKO (Glostrup, Denmark). The staining conditions were according to the company's recommendations and as described in a previous report.[12] A 4-μm section of paraffin-embedded cancer tissue was dewaxed in the usual manner, and the antigen retrieval method modified by our colleague was applied to enhance the immunoreactivity of the protein in the paraffin sections.[13] In brief, the sections were boiled in 0.01 *M* citrate buffer (pH 6.0) for 15 min by microwave treatment, and after a pretreatment in methanol with 0.3% hydrogen peroxide for 15 min to inactivate endogenous peroxidase activity, the sections were rinsed with PBS. The sections were incubated with the primary antibody for EPHA2 at room temperature for 1 h in a moist chamber, and then with Universal Immuno-peroxidase Polymer, *N*-Histofine, and Simple Stain MAX PO (Nichirei, Tokyo) for 30 min at room temperature. The first colorization was performed with 0.06 mmol/liter 3,3′-diaminobenzidine (DAB) and 2 mmol/liter hydrogen peroxide in 0.05 mol/liter Tris-HCl buffered at pH 7.6 for 10 min. The sections were again boiled with 0.01 *M* citric acid buffer for 15 min, incubated with the primary antibody for CD34 at room temperature for 1 h in a moist chamber, and then reacted with Universal Immuno-peroxidase Polymer, *N*-Histofine, Simple Stain MAX PO (Nichirei) for 30 min at room temperature. The sections were rinsed with PBS three times after every step of incubation. After the second colorization, with 3-amino-9-ethylcarbazole, nuclear counterstaining with hematoxylin was performed. A section without the primary antibody was included in each experiment as a negative control. Absorption by the peptides used for immmunization (supplied by the same company) was used to confirm immunoreactive specificity. The conditions of the staining procedures were optimized for each antibody (Table 2).

As to invasion depth, our cases contained 3 sm, 3 mp, 31 ss cases, and no m cases, thus a comparison including m and sm was not expected to be necessarily valid. So, 25 cases of adenoma containing focal cancer (all with invasiveness of m and sm), 6 to 25 mm in size were recruited and double-stained to evaluate EPHA2 expression in the earliest stage of colorectal carcinogenesis.

Microvessel detection and counting. The method of microvessel detection and counting has been described previously.[14] Briefly, the microvessels in the tumors and surrounding area were visualized with anti-CD34 Mab diluted 1:100, after heating the sections in a microwave oven twice for 5 min at 700 W in citrate buffer (pH 7.6). Biotinylated anti-mouse IgG was applied for immunoreaction, followed by colorization by means of the streptavidin-biotin method. A single microvessel was defined as any brown, immunostained endothelial cell that was separated from adjacent microvessels, tumor cells, and connective tissue elements. The microvessel count (MVC) was determined independently by two pathologists in every case. The procedure for evaluation of the MVC included selection of the three representative areas where rather dense neovascularization was detectable under low microscope power (×10 objective lens and ×10 ocular lens) and then counting vessels at higher magnification (×20 objective lens and ×20 ocular lens). The average of the counts in three fields was recorded. Large vessels with thick, muscular walls were excluded from the MVC. Each pathologist evaluated the slides without any knowledge of the radioactivity count or vessel count made by the other pathologist. Clinical information was also concealed from the pathologist when the vessels were counted. No significant differences in MVCs were found between the two observers, and the results obtained by the two observers were very highly correlated (data not shown). We used the mean values for the subsequent analysis. MVCs were categorized as a dichotomous variable (low MVC vs. high MVC), and a count of 192 microvessels (mean+1 SD) was used as the cut-off point in the dichotomous categorization to distinguish the low-MVC group from the high-MVC group.

Statistical analysis. The statistical significance of the associations between the expression of EPHA2 and EFNA1 in normal and tumor tissues was evaluated by use of the Wilcoxon signed-rank test, which is based on the rank of the differences between each pair of tissues examined. The results were considered significant when the *P* value was less than 0.05. The statistical significance of the expression of EPHA2/EFNA1 in tumor tissue according to clinical stages or tumor size was evaluated by two-way analysis of variance. The sample correlation coefficient was used for the comparisons (Fig. 4). The results were considered significant when the *P* value was less than 0.05. All statistical procedures were performed with SAS software package (version 8.2, SAS Inc., 2001).

Results

Semi-quantitative RT-PCR analysis. EPHA2 and EFNA1 were more highly expressed in tumor tissue than in normal tissue in

22 (59.5%) of the 37 cases and 25 (67.5%) of the 37 cases, respectively. RT-PCR quantitative analysis revealed statistically significant overexpression of EFNA1 in tumor tissues compared to normal tissue, but not for EPHA2 (for EFNA1, P=0.009; for EPHA2, P=0.100). Simultaneous overexpression of EPHA2 and EFNA1 in tumor tissue compared to normal tissue was found in 15 (40.5%) out of the 37 specimens. Overexpression of EPHA2 was detected in 20 (60.6%) of the 33 cases of differentiated adenocarcinoma and in two (50%) of the four cases of non-differentiated adenocarcinoma. Overexpression of EFNA1, on the other hand, was detected in 21 (63.6%) of the 33 cases of differentiated adenocarcinoma and in four (100%) of the four cases of non-differentiated adenocarcinoma. Both EPHA2 and EFNA1 overexpression was detected in the two early cases,. EPHA2 overexpression in tumors compared to the corresponding normal portions was detected in 15 (71.4%) of the 21 stage I and II tumors vs. seven (43.8%) of the 16 stage III and IV tumors. EFNA1 overexpression was detected in 15 (71.4%) of the 21 stage I and II tumors vs. ten (62.5%) of the 16 stage III and IV tumors. In summary, overexpression of both EPHA2 and EFNA1 in tumors was more prominent in stages I and II than in stages III and IV (P=0.007 for EPHA2, P=0.007 for EFNA1). Moreover, two out of the three cases of distant metastasis showed markedly reduced expression of EPHA2 in the tumor tissue and overexpression of EFNA1. Representative results for β-actin, EPHA2, and EFNA1 in the colorectal cancer specimens are shown in Fig. 1, A and B. When we analyzed their expressions according to tumor size, EPHA2 overexpression was detected in 15 (71.4%) of the 21 tumors less than 5 cm in size, as opposed to seven (43.8%) of the 16 tumors larger than 5 cm. Similarly, overexpression of EFNA1 was detected in 16 (76.2%) of the 21 tumors smaller than 5 cm, as opposed to eight (50%) of the 16 tumors larger than 5 cm. Thus, overexpression of both EPHA2 and EFNA1 was more marked in smaller tumors (P=0.017 for EPHA2, P=0.001 for EFNA1). In other words, tumors of less than 5 cm in stages I and II had a significantly greater probability of EPHA2 and EFNA1 overexpression (P=0.001 for EPHA2, P=0.001 for EFNA1; two-way-analysis of variance, Fig. 2, A and B). The expression profiles of EPHA2 and EFNA1 are shown in Table 3.

Immunohistochemistry of CD34 with EPHA2 (MVC high, MVC low). The numbers of microvessels, were calculated as the mean of the count in three inspected areas that had been selected from a larger preview area that exhibited the most intense vascularization (range: 51–313 vessels in colorectal cancer; 38–217 vessels in adenoma with focal cancer). To verify the correlations between EFNA1 and EPHA2 overexpression and microvessel count *in situ*, double-staining with antibodies to EPHA2 and CD34 was also performed. High MVCs were noted in the areas where overexpression of EPHA2 was marked (Fig. 3A). Overexpression of EPHA2 was also noticed in focal cancer cells surrounding microvessels in the polyps (Fig. 3B). Correlation coefficients were calculated for MVC and expression of EPHA2 and EFNA1 and they are shown in Fig. 4, A and B respectively (for EPHA2, r=0.343, P=0.037; for EFNA1, r=

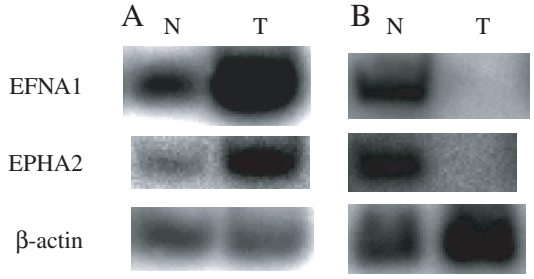

Fig. 1. Semi-quantitative RT-PCR in colorectal cancer. Expressions of EFNA1, EPHA2, and β-actin in colorectal cancer (N, normal tissue; T, tumor tissue). (A) A stage II, ascending colon cancer. Both EPHA2 and EFNA1 are overexpressed in the tumor portion compared with the normal tissue. (B) A stage IV, ascending colon cancer with lung metastasis. Both EPHA2 and EFNA1 expression are downregulated in the tumor portion compared with the normal tissue.

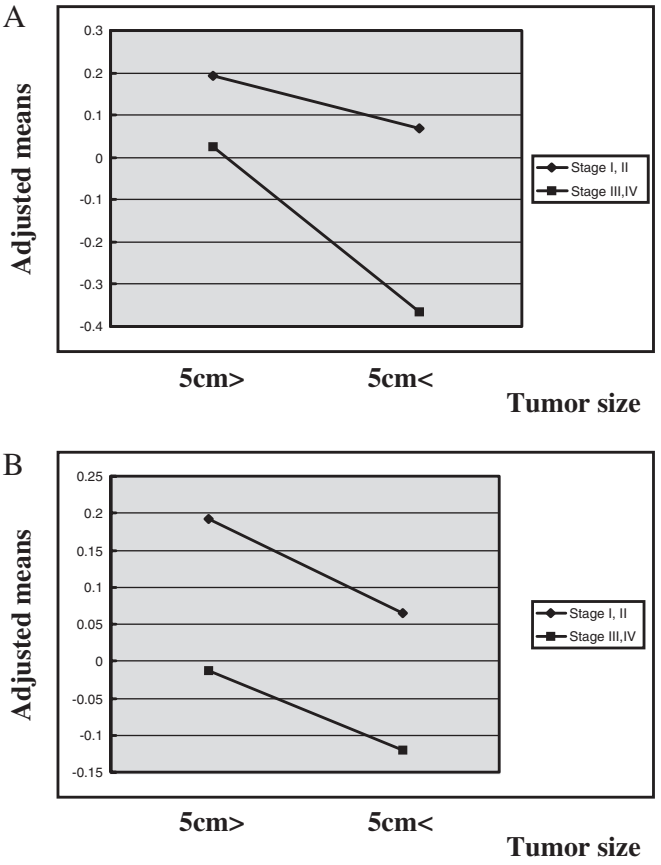

Fig. 2. (A) Cases of stage I and II tumor less than 5 cm in size show significantly higher EPHA2 expression (P=0.001). (B) Cases of stage I and II tumor less than 5 cm in size show significantly higher EFNA1 expression (P=0.001). Possible association between stage, size, and EPHA2 or EFNA1 was examined by analysis of variance. Adjusted means are the least-squares means as a result of two-way analysis of variance. A diamond and a square indicate Stage I, II and Stage III, IV respectively.

Table 2. Immunohistochemical staining methods

Antibody	Provider	Epitope retrieval	Dilution	Incubation
pAB, SC-924, EPHA2	Santa Cruz	15 min MW in citrate buffer (pH 6) at 500 W	1:100	1 h, RT
pAB, QBEnd10, CD34	DAKO	15 min MW in citrate buffer (pH 6) at 500 W	1:100	1 h, RT

MW, microwave treatment; RT, room temperature.

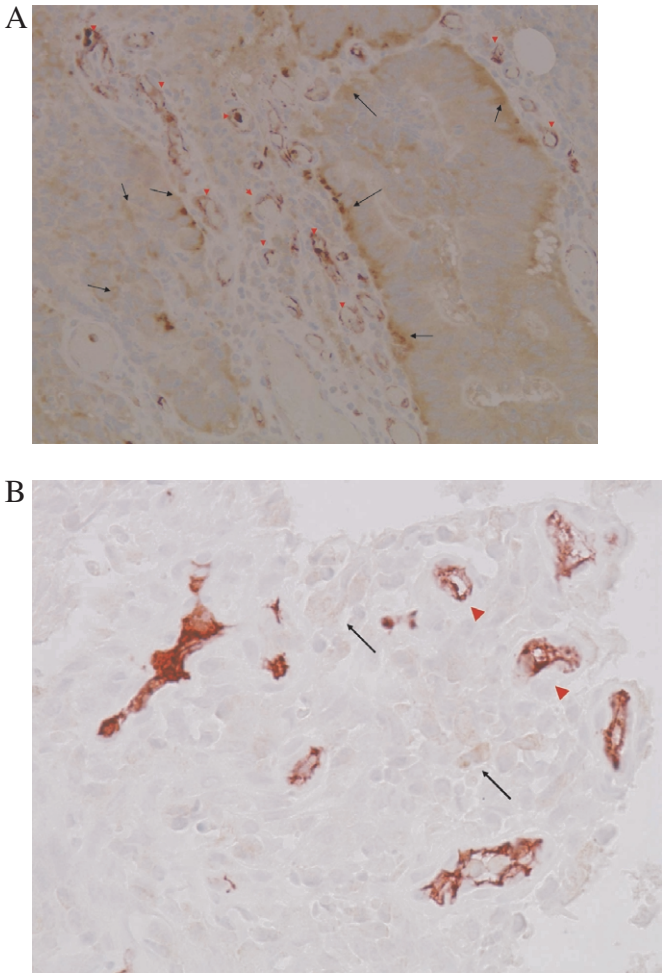

Fig. 3. Double-immunostaining of EPHA2 and CD34. (A) MVC high: In these cases, EPHA2 is overexpressed in tumor cells (arrows, brown color by DAB) compared with normal tissues as determined by semi-quantitative RT-PCR analysis. Note the the high density of the vessels identified with CD34 (arrowheads, purple color). (B) MVC in focal cancer in adenoma. Clusters of cancer cells expressing EphA2 (arrows, brown color) are near the microvessels (red arrowheads, purple color). Original magnifications, ×400.

−0.165, P=0.3313). MVC in adenomas with focal cancer and in colorectal cancer at each stage were recorded, and massive induction of MVC was not apparent in focal cancer with adenoma (data not shown). Histologically, heterogeneity of EPHA2 expression was seen (data not shown), but the relationship to the tumor location (depth) was not strictly evaluated.

Discussion

In the present study, we used semi-quantitative RT-PCR analysis and microvessel counts after immunostaining of CD34 to evaluate EPHA2/EFNA1 expression levels and their possible relation to microvessel induction in 37 cases of colorectal cancer. Several factors, including vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF), have already been well documented in colon cancer from the standpoint of their contribution to tumor-related biological phenomena, including tumor cell invasion and tumor-induced neovascularization.[15, 16] However, there are many other RTKs and ligands that may play a critical role in the biological character of human colorectal cancer, and information on them and their significance in the natural and clinical settings is still sparse.[17]

EPH receptors represent the largest known family of RTKs, and they are activated by interaction with cell-surface ligands, called ephrins (EFNs). High levels of EPHA2 RTK have been reported, especially, in advanced melanoma and prostate cancer,[18, 19] and *in vitro* evidence for EPHA2's role in tumorigenesis has been reported in MCF-10A, a mammary epithelial cell system.[9] EPHA2 binds five different ligands, EFNA1-5, which are attached to the cell membrane.[20] The gene encoding EFNA1, the first identified ligand for EPH receptor, has been cloned from human umbilical vein endothelial cells (HUVECs) as a gene induced by TNF-α.[21, 22] Easty *et al*. investigated possible involvement of EFNA1 in human cancer progression, and found increasing expression of EFNA1 as melanoma progressed.[22] In an investigation of human colon cancer, on the other hand, immunohistochemical methods have shown that Caco-2, a cell line derived from human colon cancer, contains both EPHA2 and EFNA1 within the same cells, and the authors of that study claim that an autocrine loop of these molecules exists in these cells.[23] Potla *et al*. reported that forced reduction of EFNA1 expression in HT29, another colon cancer cell line, restrained its three-dimensional growth,[24] and another line of investigation[7] has suggested that expression of EFNA1 on the vasculature may influence the attachment of metastatic cells expressing EPH receptors. Our immunohistochemical search revealed no immunoreactivity of EPHA2 in the vessels, that is, no mixed coloration (brown and purple) was detected.

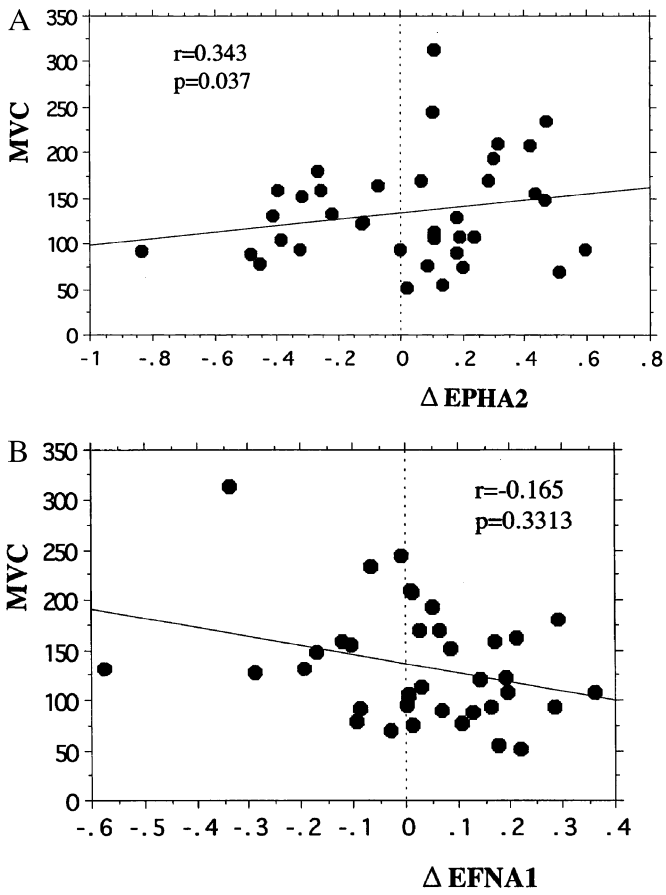

Fig. 4. MVC obtained after immunostaining with anti-CD34. A count of 192 was used as the cut-off point to divide cases into low and high MVC groups. (A) EPHA2 is significantly correlated with MVC (r=0.34, P=0.037). (B) The correlation of EFNA1 with MVC is not significant (r=−0.16, P=0.33).

Table 3. Overexpression of EPHA2 and EFNA1 in 37 colorectal cancer patients

Clinical factors		No. of cases of EPHA2 overexpression		No. of cases of EFNA1 overexpression	
Total cases	(n=37)	22 (59.5%)	P=0.100*	25 (67.5%)	P=0.009*
TNM classification					
stages I, II	(n=21)	15 (71.4%)	P=0.007*	15 (71.4%)	P=0.007*
stages III, IV	(n=16)	7 (43.8%)	P=0.804*	10 (62.5%)	P=0.454*
Tumor size					
5 cm>	(n=21)	15 (71.4%)	P=0.017*	16 (76.2%)	P=0.001*
5 cm<	(n=16)	7 (43.8%)	P=1.000*	8 (50%)	P=1.000*

The significance of the overexpression of EPHA2 and EFNA1 in tumor vs. normal tissues was evaluated by use of the Wilcoxon signed-rank test.

It is noteworthy that our data clearly indicate that colorectal cancer expresses EPHA2 and EFNA1 significantly more abundantly than normal tissue. In our cases, EPHA2 and EFNA1 expression seems to be associated with clinicopathological parameters. Both messages are more significantly overexpressed in stages I and II than in stages III and IV, and they are more overexpressed in tumors less than 5 cm in size than in those larger than 5 cm. These data suggest the importance of EFNA1 and EPHA2 in the early step of colorectal cancer progression.

Angiogenesis is thought to be critical to the course of colorectal cancer development. Peri- and intra-tumoral angiogenesis, which we were able to evaluate by counting the microvessels, has long been considered a possible surrogate indicator of tumor relapse, liver metastasis or hematogenous metastasis, and eventually of the overall survival of the colorectal cancer patients.[25–28] It is well known that some EPH receptors and ligands are intricately committed to vasculogenesis in embryonic development. Our present finding of high microvessel density in EPHA2-positive tumor areas seems to support the hypothesis that EPHA2 is important to the growth of colorectal cancer through angiogenesis in the earlier stages. EFNA1 itself is also a melanoma growth factor, and it is angiogenic and a chemoattractant for endothelial cells.[22] TNF-α-induced EFNA1 expression is mediated through the JNK and p38 MAPK signaling pathways,[29] and recent data suggests that EFNA1-induced cytoskeletal reorganization requires FAK and p130^{cas}.[30] Interestingly, contrary to our expectation, high MVCs were more strongly correlated with the level of expression of EPHA2 than with that of EFNA1. More recent data demonstrating that blocking of EPHA receptor activation inhibits angiogenesis in two different animal tumor models[31] are also consistent with our observation.

A previous study suggested that strong EFNA1 immunoreactivity in tumors is significantly associated with decreased survival, and EPHA2 has been found to be associated with increased tumor cell proliferation (Ki-67 positivity) in malignant melanoma.[12] More recently, a subset of EPHA2 monoclonal antibodies has been discovered to inhibit soft agar colonization, suggesting that the antibodies repress malignant behavior by down-regulating EPHA2.[32] Cheng *et al*. suggested that interaction between EPHA2 and EFNA1 ligand is necessary for induction of maximal neovascularization by VEGF.[33] EPHA2 is activated by VEGF through induction of EFNA1 ligand. Actually, a soluble EPHA2-Fc receptor inhibits VEGF-induced endothelial cell survival, migration, sprouting, and corneal angiogenesis. On the other hand, overexpression of EFNA1 may induce EPHA2 degradation.[34] Thus, it is possible that the contributions of EPHA2 and EFNA1 to MVC may be different. Taken together, our data suggest that EPHA2 might be a candidate target for clinical treatment of colorectal cancer and angiogenesis.

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