Ephrin-A1 mRNA is associated with poor prognosis of colorectal cancer

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Abstract. We previously studied hypoxic tumor cells from hepatic metastases of colorectal cancer (CRC) and determined several potential prognostic factors, including expression of ephrin-A1 (EFNA1), which was highly induced by hypoxia. Here, we further evaluated the prognostic impact of EFNA1 expression. Samples from a total of 366 CRC patients from 11 institutes were analyzed by either microarray (n=220) or quantitative reverse-transcriptase polymerase chain reaction (n=146). EFNA1 was an independent prognostic factor for CRC ($p<0.05$). *In vitro* assays revealed that loss of EFNA1 following siRNA treatment was associated with reduced proliferative activity and decreased invasion and migration of CRC cell lines. EFNA1 expression is a useful marker for predicting high risk of relapse and cancer-related death in patients who have undergone curative resection for CRC.

Introduction

Colorectal cancer (CRC) is one of the most common human malignancies worldwide. Despite recent advances in treatment with chemotherapy and with biologic agents such as bevacizumab or cetuximab (1), CRC is still a major cause of cancer death (2). Furthermore, the indications for these therapies have been limited due to side-effects and the small number of known target genes (3). Thus, there is a crucial need to explore novel cancer-related genes that may serve as diagnostic markers and molecular targets in CRC therapy.

Hypoxia is a main feature of cancer, with intratumoral hypoxia affecting every major aspect of cancer biology, including cell invasion, metastasis, and cell death (4). In tumor cells under hypoxia, hypoxia inducible factor 1 (HIF-1) plays a critical role in promoting the expression of hypoxia-response genes that are associated with an aggressive tumor phenotype (5-7). These hypoxia-related genes include several angiogenic factors, such as vascular endothelial growth factor (VEGF), that play important roles in cancer biology. The anti-VEGF antibody bevacizumab is used clinically for treatment of several human cancers (8), supporting the use of hypoxia-induced genes as clinically relevant therapeutic targets.

Ephrin-A1 (EFNA1) is known as an angiogenesis factor, and is induced through a HIF-dependent pathway (9,10). EFNA1 was originally isolated as a secreted protein in conditioned media from cultures of human umbilical vein endothelial cells treated with tumor necrosis factor-α (11), and the gene was found to be induced by tumor necrosis factor-α in these cells (12). EFNA1 expression has also been observed in tumor endothelial cells and tumor cells, and shown to induce endothelial cell migration (13), capillary assembly *in vitro*, and corneal angiogenesis *in vivo* (14). EFNA1 and its receptor, Eph receptor 2 (EphA2), are associated with carcinogenesis, angiogenesis (13,15-17), and tumorigenesis in various cancers, including urinary bladder carcinoma (18), breast cancer (19,20), gastric cancer (21), glioma (22), and malignant mesothelioma (23).

Previously, we detected several potential prognostic factors and therapeutic targets in hypoxic tumor cells from hepatic metastases of CRC *in vivo* (24). In these experiments, Ephrin-A1

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Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction

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gene (*EFNA1*) expression was highly induced in hypoxic regions of liver metastases (fold-change = 1.58, p=0.005). Thus, we hypothesized that *EFNA1* expression may be a novel prognostic factor in CRC patients. In the present study, we examined the correlation between *EFNA1* expression and prognosis in CRC patients, and we analyzed the biological significance of *EFNA1* expression in human CRC.

Materials and methods

Cell culture. The colon carcinoma cell lines DLD1, Lovo, HCT116, HT29, SW480, and CaCo2 were obtained from the American Type Culture Collection. KM12sm was a kind gift from Professor T. Minamoto (Cancer Research Institute, Kanazawa University, Japan). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37˚C in a humidified incubator with 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were grown on MCDB131 culture medium (Chlorella Inc., Tokyo, Japan) supplemented with 10% fetal bovine serum, antibiotics, and 10 ng/ml fibroblast growth factor. For culture under hypoxic conditions, cells were grown for up to 72 h at 37˚C in a continuously monitored atmosphere of 1% O_2, 5% CO_2, and 94% N_2 using a multigas incubator (Model 9200, Wakenyaku Co., Kyoto, Japan). Control cells were cultured under normoxic conditions (21% O_2).

Patients and clinical sample collection. For microarray analysis, we prospectively collected 220 primary CRC samples from consecutive patients who had curative operations between 2003 and 2006 at Osaka University Hospital and its nine associated hospitals. For quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), tumor samples were consecutively collected from 146 CRC patients who had curative surgery from 1993 to 2002 at the Department of Surgery, Medical Institute of Bioregulation, Kyushu University. None of the included patients had preoperative chemotherapy or irradiation. After surgery, patients with stage III/IV tumors were treated with 5-fluorouracil (5-FU)-based chemotherapy. The Human Ethics Review Committee of Osaka University and Kyushu University approved the use of the resected samples.

Immediately after surgical resection, a piece of each primary colorectal cancer tissue sample was collected from the fresh specimens, and stored in RNA Stabilization Reagent (RNA Later; Ambion, Inc., Austin, TX, USA) at -80˚C until RNA extraction.

RNA extraction and real-time quantitative RT-PCR analysis. Total RNA was extracted with a single-step method, using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD, USA) at Osaka University and Isogen (Nippon Gene, Tokyo, Japan) at Kyushu University. Complementary DNA (cDNA) was generated using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). Real-time monitoring of PCR reactions was performed using the LightCycler™ system (Roche Applied Science, Indianapolis, IN, USA) for quantification of mRNA expression, as described previously (25). The housekeeping gene porphobilinogen deaminase (PBGD) (26,27) was used as an internal standard. The sequences of PBGD primers were as follows: sense primer, 5'-AACGGCGGAAGAAACAG-3' and antisense primer, 5'-TCCAATCTTAGAGAGTGCA-3'. The *EFNA1* primer sets were designed to flank one intron, and were tested to ensure amplification of only cDNAs to avoid amplification of possibly contaminating genomic DNA. The sequences of these PCR primers were as follows: *EFNA1* sense primer, 5'-TGCCGTCCGGACGAGACAGGC-3' and *EFNA1* antisense primer, 5'-CTGGAGCCAGGACCGGGACTG-3'.

Immunohistochemistry. Immunohistochemical analysis was performed as described previously (28). Frozen sections (4 μm) were fixed in 4% paraformaldehyde for 5 min. The slides were incubated with anti-EFNA1 rabbit polyclonal antibody (1:200; Abcam, Cambridge, UK) overnight at 4˚C. Negative control sections were incubated with normal rabbit serum instead of the primary antibody. All slides were evaluated in a blinded manner by a pathologist.

Western blot analysis. Western blot analysis was performed as we previously described (29). To detect EFNA1 protein expression, extracted protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blot analysis using the EFNA1-specific antibody (1:500; Abcam).

Transfection reagents. *EFNA1* siRNA was purchased from Invitrogen (Carlsbad, CA, USA) and control negative siRNA was purchased from Qiagen Inc. (Valencia, CA, USA). siRNA sequences for *EFNA1* and for the irrelevant control were as follows: *EFNA1* siRNA #1, 5'-CCAUACAUGUGCAGCUGA AUGACUA-3'; *EFNA1* siRNA #2, 5'-CAGAGGUGCGGG UUCUACAUAGCAU-3'; and control negative siRNA, 5'-AAT TCTCCGAACGTGTCACGT-3'. CRC cell lines were transfected with siRNA using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

Cell proliferation assay. Cell growth was measured by adding WST-1 reagent (Roche) and incubating at 37˚C for 2 h. Absorbance was measured at 450 nm with background subtraction at 630 nm. Results were given as the mean ± SD of five separate experiments.

In vitro migration/invasion assay. The invasion assay was performed using transwell cell culture chambers (BD Biosciences, Bedford, MA, USA) as described previously (24). Briefly, colorectal cancer cells at a concentration of 20,000 cells/ml were placed in the top chamber of a two-chamber assay system and incubated for 24 h with and without 10% FBS placed in the lower chamber. After 48 h, cells that had invaded the undersurface of the membrane were fixed with 100% methanol and stained with 1% toluidine blue. Four microscopic fields were randomly selected for cell counting.

Cell migration assays were performed using BD Falcon cell culture inserts containing polyethylene terephthalate membranes (8-μm pore size) from BD Biosciences. Similar to the invasion assays, the cells were placed in the top of the chamber, while 10% FBS was added to the lower chamber. Results were given as the mean ± SD of four separate experiments.

Tumor cell and endothelial cell co-culture migration assays. The co-culture migration assay was performed using BD Falcon cell culture inserts containing collagen type IV (3-μm pore size) according to the manufacturer's instructions (BD Biosciences). Briefly, HUVECs at a concentration of 20,000 cells/ml were placed in the top chamber of a two-chamber assay system and incubated for 24 h, while HCT116 colorectal cancer cell lines transfected with control negative siRNA or *EFNA1* siRNA were placed in the lower chamber. After the incubation period, the cells on the upper side were removed using a cotton swab; then the coated filters were removed. The undersurface of the membrane was fixed with 100% methanol and stained with 1% toluidine blue. HUVEC migration was quantified microscopically by counting the cells that had migrated into the filters. Results were given as the mean ± SD of four separate experiments.

Statistical analysis. For clinicopathological analyses, study samples were divided into high- and low-expression groups based on the median EFNA1 mRNA expression levels in tumor tissue. All statistical analyses were carried out using the StatView J-5.0 program (Abacus Concepts, Inc., Berkeley, CA). The post-operative period was measured from the date of surgery to the date of the last follow-up or death. Differences were estimated using Fisher's exact probability test. Survival curves were calculated by the Kaplan-Meier method, and compared statistically using the log-rank test. To estimate relative risk (RR) and 95% confidence interval (95% CI), univariate and multivariate analysis were performed using the Cox proportional hazards regression model. Data are reported as mean ± SD. Mean values were compared using the Mann-Whitney test. A probability value of <0.05 was deemed to be statistically significant.

Results

Patient profiles. The patients selected for microarray analysis included 131 males (59.5%) and 89 females (40.5%). The primary tumor was in the colon in 141 patients and in the rectum in 79 patients; 98.2% of tumors were well or moderately-differentiated adenocarcinomas, and 4 patients (0.8%) had poorly-differentiated adenocarcinomas. In regards to TNM staging, 109 patients (49.5%) were stage I or II and 111 patients (50.5%) were stage III or IV. Detailed information is shown in Table I.

Table I. Clinicopathological factors of CRC patients analyzed by microarray.

	High EFNA1 expression	Low EFNA1 expression	p-value
Age at surgery (years)			
>67	60	62	0.787
≤67	50	48	
Gender			
Male	66	65	0.891
Female	44	45	
Tumor site			
Colon	66	75	0.212
Rectum	44	35	
Depth of tumor invasion			
T1, 2	12	18	0.246
T3, 4	98	92	
TNM stage			
I or II	52	57	0.498
III or IV	58	53	
Lymph node metastasis			
Present	57	52	0.589
Absent	53	58	
Venous invasion			
Present	70	61	0.272
Absent	40	49	
Histological type[a]			
Differentiated	106	110	-
Undifferentiated	4	0	

[a]Differentiated type included well and moderately-differentiated adenocarcinoma. Undifferentiated type included poorly-differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma.

The CRC patients analyzed by qRT-PCR included 83 males (56.8%) and 63 females (43.2%). The primary tumor site was the colon in 92 patients (63.0%), and 8.9% of patients had poorly-differentiated adenocarcinoma or mucinous adenocarcinoma. Detailed information is shown in Table II. As shown in Tables I and II, high expression and low expression were divided based on the median value in each assay. No significant differences were observed in the clinicopathological factors between high and low *EFNA1* expression groups in the two data sets (Tables I and II).

Survival analysis stratified by EFNA1 mRNA expression. Kaplan-Meier survival curves demonstrated that patients with high *EFNA1* expression showed significantly shorter survival than those with low *EFNA1* expression, in terms of both disease-free survival (DFS) in microarray data (Fig. 1A) and cancer-related survival (CRS) in qRT-PCR data (Fig. 1B). Next, we performed univariate analysis of clinicopathological factors and found that lymph node metastasis, venous invasion, tumor differentiation, depth of tumor invasion, and *EFNA1* expression were significantly associated with DFS based on microarray data and CRS based on qRT-PCR data (Tables III and IV). Multivariate Cox regression analysis revealed that *EFNA1* expression and lymph node metastasis remained independent prognostic factors (Tables III and IV).

EFNA1 expression in CRC cell lines and colorectal tumor tissue. Immunohistochemistry of the CRC tissue samples showed that tumor cells expressed EFNA1 mainly at the plasma membrane (Fig. 2), while normal colonic epithelium scarcely expressed

Table II. Clinicopathological factors of CRC patients analyzed by qRT-PCR.

	High EFNA1 expression	Low EFNA1 expression	p-value
Age at surgery (years)			
>66	43	46	0.367
≤66	30	27	
Gender			
Male	43	40	0.371
Female	30	33	
Tumor site			
Colon	42	50	0.126
Rectum	31	23	
Depth of tumor invasion			
T1	19	25	0.185
T2, 3, 4	54	48	
TNM stage			
I or II	33	43	0.072
III or IV	40	30	
Lymph node metastasis			
Present	33	28	0.502
Absent	40	45	
Venous invasion			
Present	13	15	0.838
Absent	60	58	
Histological type[a]			
Differentiated	69	66	0.275
Undifferentiated	4	7	

[a]Differentiated type included well and moderately-differentiated adenocarcinoma. Undifferentiated type included poorly-differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma.

EFNA1. Western blot analysis showed that the EFNA1 protein was expressed in the seven CRC cell lines tested (Fig. 3A).

EFNA1 mRNA overexpressed in CRC cell lines under hypoxia. Fig. 3B shows the EFNA1 mRNA expression in four CRC cell lines. EFNA1 mRNA was progressively induced with hypoxia in all CRC cell lines examined, and highly expressed after 48 h under hypoxia in HT29, DLD1, and Lovo. On the other hand, EFNA1 mRNA in HCT116 was expressed after 6 h of hypoxia.

Effects of EFNA1 on growth, invasion, and migration of CRC cells. To assess the potential relevance of EFNA1 as a therapeutic target, *in vitro* knockdown experiments were performed in HCT116. Western blot analysis showed moderate and strong reductions in EFNA1 after treatment with siRNA #1 and

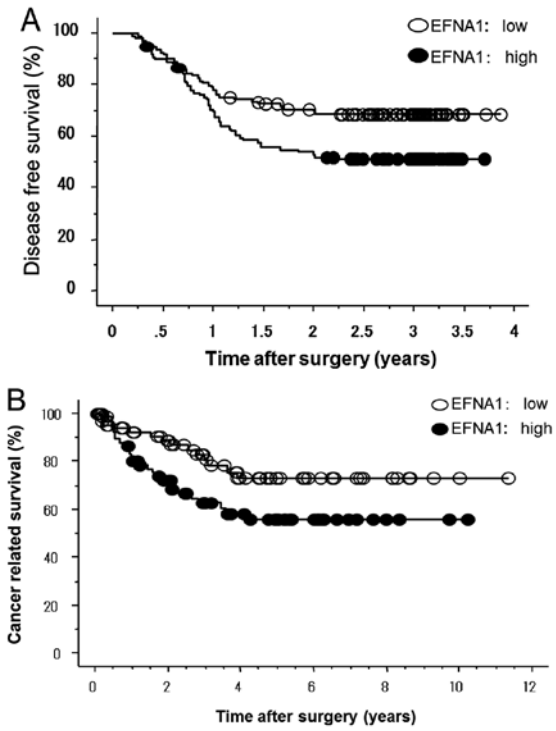

Figure 1. Kaplan-Meier survival curves of colorectal cancer patients according to *EFNA1* mRNA expression. Patients in the high *EFNA1* mRNA expression group had poorer survival than those in the low expression group, as shown by microarray analysis data (p=0.011) (A) and RT-PCR analysis (p=0.029) (B).

Table III. Univariate and multivariate analyses of the relationships between clinicopathological factors and disease-free survival in microarray data.

Factor	p-value	OR	95% CI	p-value
Lymph node metastasis	<0.001	2.393	1.489-3.848	<0.001
Venous invasion	<0.001	1.801	1.073-3.002	0.026
EFNA1 expression	0.011	1.586	1.026-2.452	0.038
Tumor differentiation	0.008	3.868	1.515-9.874	0.005
Depth of invasion	0.009	1.681	0.585-4.828	0.335
Tumor site	0.069			
Age	0.143			

Table IV. Univariate and multivariate analyses of the relationships between clinicopathological factors and cancer-related survival in qRT-PCR data.

Factor	p-value	OR	95% CI	p-value
Lymph node metastasis	<0.0001	3.344	1.707-7.769	0.0008
Venous invasion	0.0005	1.784	0.942-3.585	0.0744
EFNA1 expression	0.0288	2.037	1.026-3.889	0.0417
Tumor differentiation	0.0015	1.046	0.652-4.082	0.2954
Depth of invasion	0.0001	2.253	1.199-13.611	0.0242
Tumor site	0.6044			
Age	0.1434			

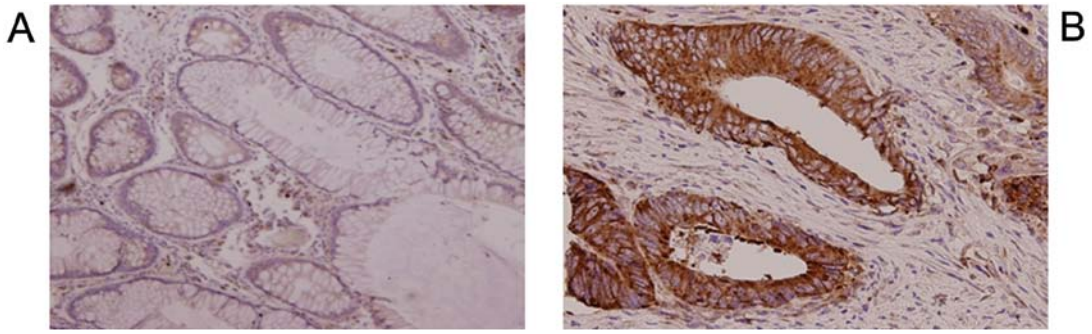

Figure 2. Immunohistochemical analysis of EFNA1 in colon cancer tissue samples. Normal colonic mucosa hardly expressed any EFNA1 (A), while colon tumor cells expressed EFNA1 on the plasma membrane (B). Magnification, x100.

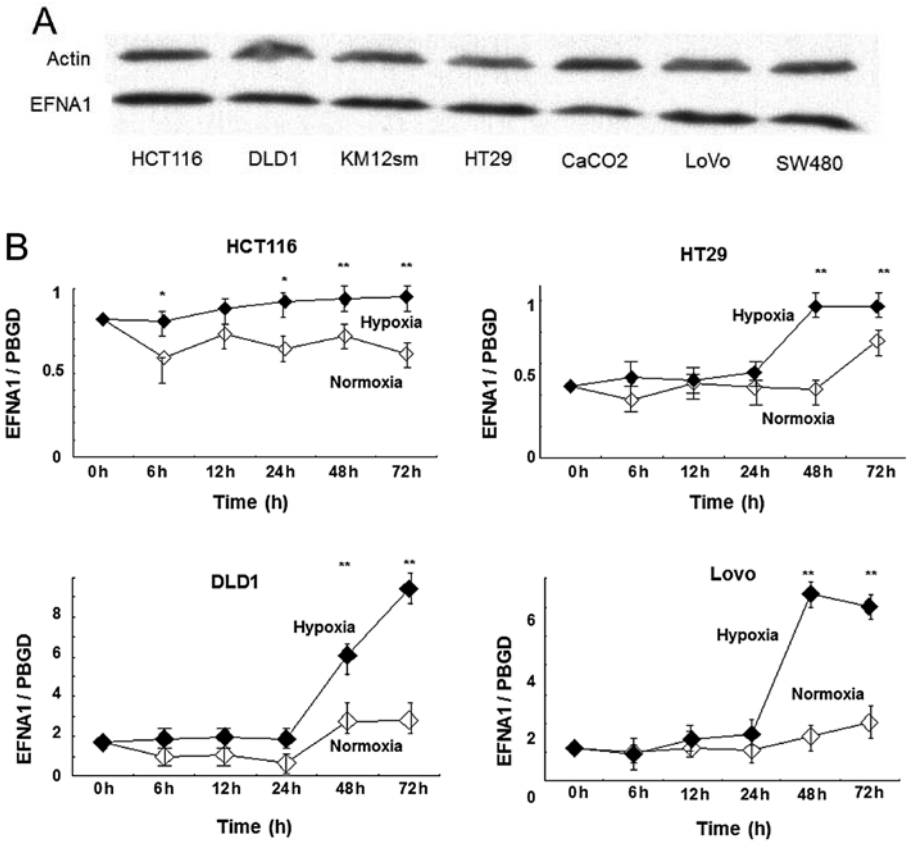

Figure 3. Analyses of EFNA1 expression. (A), Western blot analysis showed that colon cancer cell lines expressed considerable amounts of EFNA1 protein. Actin served as a control. (B), EFNA1 mRNA expressions under normoxia and hypoxia in four colon cancer cell lines. EFNA1 mRNA was induced in hypoxia in colon cancer cell lines. $^{*}p<0.05$, $^{**}p<0.01$.

siRNA #2, respectively (Fig. 4A). Significant growth inhibition was observed in siRNA #2-treated HCT116 cells ($p<0.05$, Fig. 4B). Invasion and migration assays further indicated that both siRNA #1 and siRNA #2 treatment significantly decreased the numbers of invaded and migrated cells compared to control treatments ($p<0.05$ for each; Fig. 4C). Similar results were obtained in assays with DLD1 cells (data not shown).

Effects of EFNA1 on migration of vascular endothelial cells. To examine the effects of EFNA1 on migration of vascular endothelial cells, co-cultured migration assays were performed using HCT116 cells and HUVECs. The number of migrated HUVECs was significantly smaller when cells were co-cultured with EFNA1 siRNA-transfected HCT116 cells than with negative control siRNA transfected cells ($p<0.05$; Fig. 5).

Discussion

EFNA1 expression has been previously reported to be associated with prognosis in early squamous cell cervical carcinoma (30), and *in vitro* analysis has indicated that EFNA1 expression affects growth of HT29 colon cancer cells (31). However, the prognostic impact of EFNA1 in colorectal cancer patients remains unknown. The present study evaluated the correlation between EFNA1 mRNA expression levels and prognosis in colorectal cancer patients, using microarray analysis of 220 colorectal

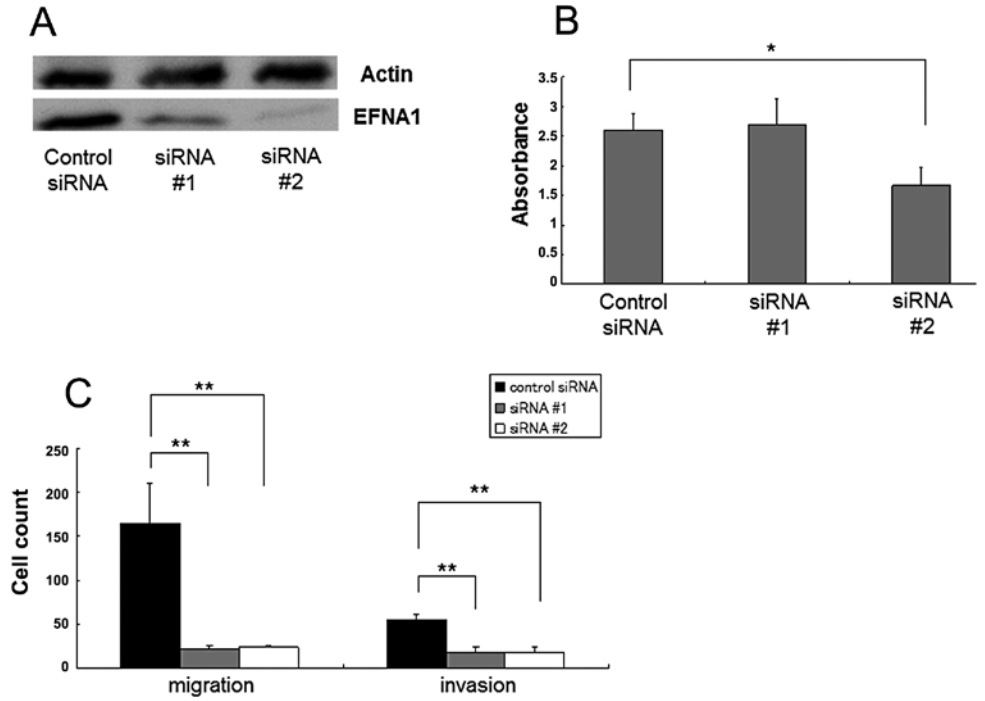

Figure 4. Correlation between EFNA1 silencing and proliferation, migration, and invasion in HCT116 cells. (A), Knock-down of EFNA1 after siRNA treatment was confirmed by western blot analysis. (B), Proliferation assay was performed in HCT116. Cell growth was inhibited at 48 h after siRNA #2 transfection, which caused the greater reduction of EFNA1 expression. (C), Migration and invasion assays showed a significant decrease in migrated and invaded cells in EFNA1 siRNA-transfected HCT116 cells.

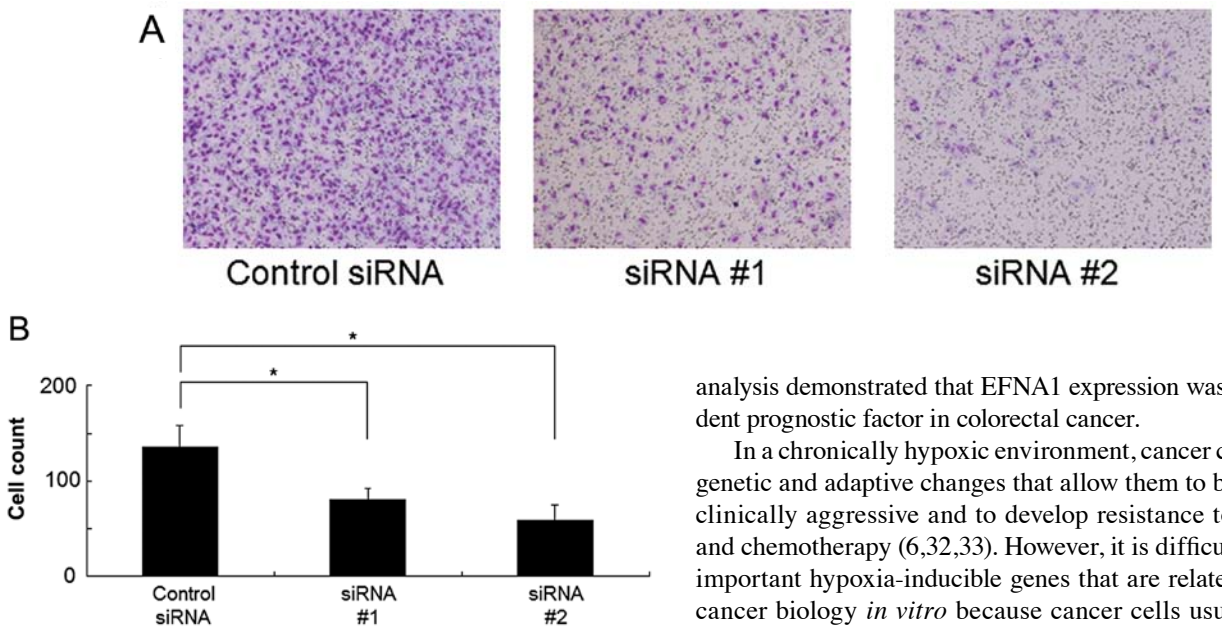

Figure 5. Effects of EFNA1 on migration of vascular endothelial cells. (A), Migrated HUVECs on the filter were stained with 1% toluidine blue and observed under a microscope. (B), A significantly smaller number of migrated HUVECs was observed following co-culture with EFNA1 siRNA-transfected HCT116 cells compared to with negative control siRNA-transfected cells. *$p<0.05$.

cancer samples and RT-PCR analysis of 146 colorectal cancer samples. The most important finding was that patients with high EFNA1 expression showed a poorer prognosis than patients with low expression in both cohorts. Furthermore, multivariate analysis demonstrated that EFNA1 expression was an independent prognostic factor in colorectal cancer.

In a chronically hypoxic environment, cancer cells undergo genetic and adaptive changes that allow them to become more clinically aggressive and to develop resistance to irradiation and chemotherapy (6,32,33). However, it is difficult to identify important hypoxia-inducible genes that are related to clinical cancer biology *in vitro* because cancer cells usually exist in chronically hypoxic conditions *in vivo* with complex interactions with several pathways.

We previously identified *EFNA1* as a candidate hypoxia-inducible gene by using tissue samples from liver metastasis of colorectal cancer (24). For finding novel hypoxia-inducible genes, the method of collecting liver metastasis samples is of particular importance. After surgical removal, the liver tissue samples were stored in OCT compound as soon as possible, usually within 10-15 min. Microarray analysis successfully identified genes that were highly induced by hypoxia *in vivo* by comparison between hypoxic tumor cells and non-hypoxic tumor cells. VEGF ranked fifth among 30,000 human genes; 10 genes

among the top 30 were well-known or relatively newly identified hypoxia-inducible genes (24). We further identified several novel hypoxia-inducible gene candidates, including *EFNA1*, and *PLOD2* (34). Based on a prospective clinical follow-up study in which the primary CRC tissues were analyzed with the same DNA chip, we focused on *EFNA1* and concluded by qRT-PCR that EFNA1 is a novel independent prognostic factor for CRC.

In the present study, we used tissue microarray to analyze not only tumor cells but also many stromal cells. Western blot analysis indicated that most CRC cell lines expressed abundant EFNA1, and the EFNA1 mRNA expression level continued to increase after 24 h in hypoxic culture condition. Immunohistochemistry showed EFNA1 protein at the plasma membrane of colon tumor cells, and as we expected, *EFNA1* was found to be associated with invasion, migration, and proliferation *in vitro*. These findings suggest that EFNA1-expressing cells have more malignant potential than cells not expressing EFNA1.

Although our data indicate that EFNA1 expression could be a prognostic marker, there was no correlation between EFNA1 expression levels and clinicopathological factors, including TNM stage. EFNA1 was previously reported to be a proangiogenic signal, facilitating angiogenesis-dependent metastatic spread (19). To investigate this possibility, we performed co-culture experiments and found that HUVEC migration was inhibited by co-culture with HCT116 cells in which EFNA1 was silenced. This finding may partly explain why EFNA1 expression is associated with poor prognosis in CRC patients.

In conclusion, the present findings strongly suggest that the *EFNA1* expression level is a useful marker for predicting a high risk for relapse and cancer-related death in CRC patients who undergo curative resection.

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