EPHA2/*EFNA1* expression in human gastric cancer

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The erythropoietin-producing hepatocellular (*EPH*)*A2* receptor, tyrosine kinase, is overexpressed and phosphorylated in several types of human tumors and has been associated with malignant transformation. A recent report, however, indicated that stimulation of the *EPHA2* receptor ligand, ephrinA1 (EFNA1), inhibits the growth of *EPHA2*-expressing breast cancer. The authors examined the expression of *EPHA2* and *EFNA1* using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) in four gastric cancer cell lines and 49 primary gastric cancer samples, as well as in normal gastric tissue. *EPHA2* was more highly expressed in tumor tissue than in normal tissue in 27 cases (55%). *EFNA1* was overexpressed in tumor tissue in 28 cases (57%). No significant correlation was detected between the expression levels and histologic features such as tumor size, age, vessel invasion, or lymph node involvement. However, *EPHA2* overexpression was more prominent in macroscopic type 3 and 4 tumors than in type 1 or 2 advanced gastric cancer. The authors observed *EPHA2* expression in three of the four gastric cancer cell lines (AGS, KATO3, and MKN74) that were examined. In one cell line, TMK1, *EPHA2* expression was barely detectable using northern blotting, RT-PCR, and western blotting. In contrast, *EFNA1* was detected in all cell lines. In the gastric cancer cell lines that endogenously expressed *EPHA2*, stimulation with ephrinA1-Fc led to decreased EPHA2 protein expression and increased EPHA2 phosphorylation. Finally, the growth of *EPHA2*-expressing cells was inhibited by repetitive stimulation with soluble ephrinA1-Fc. Taken together, these findings suggest that *EPHA2* and *EFNA1* expression may influence the behavior of human gastric cancer. (*Cancer Sci* 2005; 96: 42–47)

The erythropoietin-producing hepatocellular (*EPH*) receptors represent the largest known family of receptor tyrosine kinases and are activated by interaction with the cell-surface ligands, ephrins (*EFN*). There is evidence to suggest that some members of the *EPH* family and their *EFN* ligands are involved in angiogenesis and oncogenesis through cell adhesion, morphogenesis, capillary sprouting, and chemoattraction.[1–5] *EPH* receptors have been classified into two subfamilies, *EPHA* and *EPHB*. *EPHA* receptors bind mainly to glycosylphosphatidylinositol-anchored *EFNA* ligands, and *EPHB* receptors bind to transmembrane *EFNB* ligands. The expression of *EPH* family transcripts has been documented in some melanomas and carcinomas.[6,7] Overexpression of *EPHA2* is believed to be sufficient to confer malignant/tumorigenic potential on non-transformed mammary epithelial cells.[8] Esophageal squamous cell carcinomas that overexpress *EFNA2* have a poorer prognosis than those that do not.[9]

Gastric cancer remains a disease with a very poor prognosis, and the role of kinases in gastric cancer cells has been a focus of research. Ogawa *et al*. identified *EFNA1* and *EPHA2* expression in a very few cases of gastric cancer in 2000, but the role of these molecules has remained unclear,[10] despite an extensive survey of tyrosine kinases in gastric cancer.[11] Therefore, the authors examined the expression of *EPHA2* and *EFNA1* in gastric cancer specimens and gastric cancer cell lines using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), northern blotting, and western blotting. This is the first documented report of *EFNA1* and *EPHA2* expression in a series of gastric cancer cases. Furthermore, the authors examined the effects of *EFNA1* stimulation on cancer cell lines that endogenously express *EPHA2*.

Materials and Methods

Tissues. For RT-PCR, human gastric cancer specimens and corresponding non-tumor tissues were obtained from 49 surgical resections carried out at Hamamatsu University School of Medicine. The clinicopathologic characteristics of these patients are shown in Table 1, and are classified according to the Japanese classification system (JCS).[12] Histologically, these specimens consisted of 22 cases of well-differentiated adenocarcinoma (tubular and papillary types) and 24 cases of poorly differentiated adenocarcinoma, including the mucinous type, and three other types (two adenosquamous and one neuroendocrine). The samples consisted of six early gastric cancers (the tumor is in the submucosal and mucosal layers in the gastric wall) and 43 advanced gastric cancers (the tumor invades through the proper muscle layer of the gastric wall). According to the pathologic TNM classification, there were 18 cases at stages I and II, and 31 cases at stages III and IV. All samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA preparation was carried out. The study design was approved by the Institutional Review Board of Hamamatsu University School of Medicine (no. 12–11).

Cell cultures. Gastric cancer cell lines (KATO3, MKN74, and TMK1) were cultured in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Gibco Invitrogen, Carlsbad, CA, USA). The AGS cells were cultured in Ham's F12K medium (ICN Biomedicals, Bryan, OH, USA) supplemented with 10% FBS. The 293T human embryonic kidney cells were cultured in Dulbecco's modified eagle medium (Nissui) supplemented with 10% FBS.

RNA extraction and reverse transcription. Total cellular RNA was extracted from human tissues using the RNA extraction reagent, ISOGEN, (Nippon Gene, Tokyo, Japan) 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, USA) in a total volume of 20 μL containing Moloney murine leukemia virus reverse transcriptase (Life Technologies) and RNase inhibitor (Toyobo, Tokyo, Japan).

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Table 1. The correlation between EPHA1 and EPHA2 expression and the clinicopathologic parameters

	No.	EPHA2		EFNA1	
		Average of log T/N	*P*-value	Average of log T/N	*P*-value
Male	38	0.25406	0.162§§	0.135515	0.913§§
Female	11	–0.05075		0.152799	
Histologic type†					
tub1, 2 and pap	22	0.1897	0.995¶¶	0.218730	0.463¶¶
por 1, 2 and muc	24	0.186329		0.093821	
Others	3	0.150229		–0.778000	
Macroscopic type‡					
1 and 2	15	0.014809	0.029§§	0.124345	0.689§§
3 and 4	26	0.380546		0.058599	
Depth of tumor invasion					
T1 (m and sm)§	6	–0.30717	0.041§§	0.241157	0.738§§
T2, 3 and 4 (mp, ss, se and si)¶	43	0.254396		0.125196	
Stage					
I (IA and IB)	11	0.192919	0.298¶¶	0.179152	0.630¶¶
II	7	0.692538		0.196717	
III (IIIA and III B)	22	0.09198		0.176363	
IV	9	0.011395		–0.044150	
Lymph node metastasis					
(–)††	15	0.302749	0.483§§	0.182655	0.665§§†
(+)‡‡	34	0.133964		0.120310	
Lymphatic invasion					
ly0 and 1	31	0.271833	0.142§§	0.193581	0.514§§
ly2 and 3	18	0.035413		0.108180	
Venous invasion					
v0	17	0.134045	0.912†††	0.200282	0.866†††
v1	16	0.279166		0.120467	
v2	10	0.12316		0.140152	
v3	6	0.186497		0.016096	

†tub1, tubular adenocarcinoma, well-differentiated type; tub2, tubular adenocarcinoma, moderately differentiated type; pap, papillary adenocarcinoma; por1, poorly differentiated adenocarcinoma, solid type; por2, poorly differentiated adenocarcinoma, non-solid type; muc, mucinous adenocarcinoma; others, adenosquamous carcinoma and neuroendocrine carcinoma. ‡Except type 0, superficial, flat tumors and type 5, non-classifiable carcinomas. §This category is called 'early cancer'. ¶This category is called 'advanced cancer'. ††'N0' using the Japanese classification system (JCS).[12] ‡‡'N1, N2 and N3' using the JCS.[12] §§Student's *t*-test. ¶¶Kruskal–Wallis test. †††ANOVA.

Semiquantitative RT-PCR analysis. The semiquantitative RT-PCR method used in this study was modified from a previously described method.[13] Briefly, cDNA was diluted in water, and mixed in a final volume of 20 μL with 0.625 μmol/L of primer pairs, 1 U of Taq DNA polymerase (Roche, Basel, Switzerland), and 1 μCi of [γ-^{32}P] dCTP (Amersham Biosciences, Piscataway, NJ, USA). Amplification was carried out in a DNA thermal cycler (PC-700; ASTEC, Fukuoka, Japan). For the human β-actin (*ACTB*) control, 30 cycles were carried out, consisting of denaturation 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 extension for 10 min at 72°C. For *EPHA2* and *EFNA1*, 35 cycles were carried out, consisting of denaturation for 45 s at 94°C, primer annealing for 1 min at 59°C, polymerization for 1 min at 72°C, and final extension for 10 min at 72°C. Under these conditions, PCR was carried out during the exponential phase of amplification for *ACTB*, *EPHA2*, and *EFNA1*. A negative control was added to exclude the possibility of DNA contamination in each reaction. The integrity of the RNA obtained from the clinical samples was confirmed by determining the presence of *ACTB* mRNA in the same samples, and the 28S and 18S peaks using Agilent 2100 Bioanalyzer (Agilent Technology, Waldbrown, Germany). The sizes of *ACTB*, *EPHA2*, and *EFNA1* were 121 base pairs (bp), 260 bp, and 230 bp, respectively. The primer sequences were as follows: (a) *EPHA2*, 5′-GCAACATCCTCGTCAACAGC-3′ (sense primer) and 5′-TGGCTTTCATCACCTCGTGG-3′ (antisense primer); (b) *EFNA1*, 5′-AACAA-GCTGTGCAGGCATGG-3′ (sense primer) and 5′-CTCCACAGATGAGGTCTTGC-3′ (antisense primer); (c) *ACTB*, 5′-GCTACGTCGCCCTGGACTTC-3′ (sense primer) and 5′-AGCGGAACCGCTCATTGCCA-3′ (antisense primer). The PCR products were separated using electrophoresis on 6% polyacrylamide gels, which were then dried and autoradiography and image analysis was carried out using MacBAS (Fuji Film, Tokyo, Japan). Representative cases showing expression of *EFNA1*, *EPHA2*, and *ACTB* in gastric cancer and the corresponding non-tumor specimens are shown in Figure 1.

Statistical analysis. The autoradiographic densities were transformed into common logarithms. For statistical comparisons of the log-transformed data between two or more groups, an ANOVA was used when the variances of the groups were equal, and the Kruskal–Wallis test was used when the variances were not equal. When simply comparing two groups, Student's *t*-test was used. Levene's test was used to assess the equality of the variance for all group comparisons. Wilcoxon's signed-rank test, based on the rank of the differences between each pair of tissues in the observation, was also used. All statistical analyses were carried out using the SPSS software program version 11.5J (SPSS Japan, Tokyo, Japan). The statistical tests were two-sided, and the results were considered to be significant when the *P*-value was <0.05.

Northern blotting. Twenty micrograms of total RNA for each of *ACTB*, *EPHA2*, and *EFNA1* was separated on 1.0% denatured agarose gels, and blotted on NitroPlus (Micron Separations, Westboro, MA, USA) for 16 h. The plasmids, pAlterMAX, containing *EFNA1* and *EPHA2* cDNA (Gene Bank accession

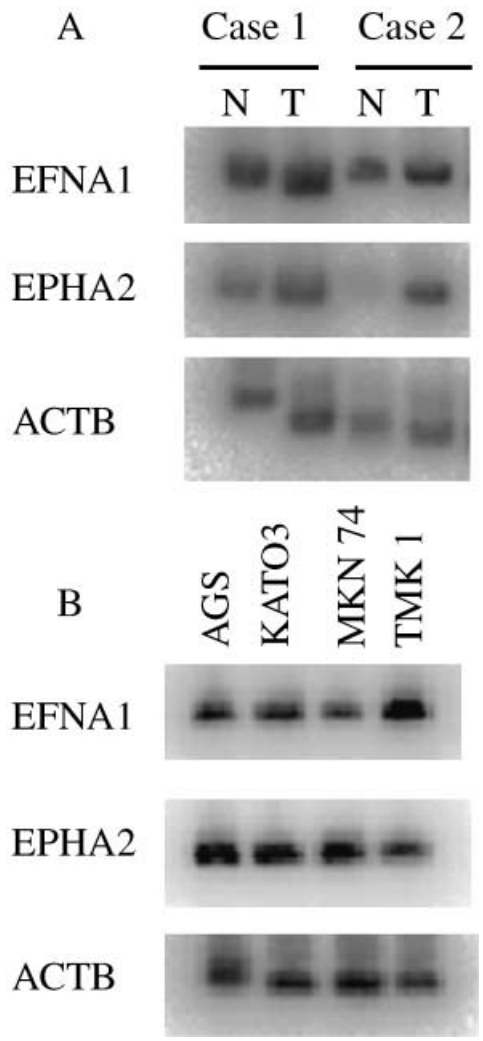

Fig. 1. Semi-quantitative reverse transcription-polymerase chain reaction data of two cases of primary gastric cancer and corresponding non-tumor tissue, and gastric cancer cell lines. (A) Both ephrinA1 (EFNA1) and EPHA2 were overexpressed in these tumors (cases 1 and 2). ACTB is shown as an internal control. (B) EFNA1 was expressed in all gastric cell lines, particularly in TMK1. EPHA2 was detected in these four cell lines, but expression was weak in TMK1 compared with the other lines. N, non-tumorous gastric tissue; T gastric cancer tissue.

numbers NM_004428 and NM_004431, respectively), have been described previously.[14] Specific probes were constructed from these plasmids using digestion, with the following appropriate restriction enzymes, *Eco*RI and *Bam*HI (for *EFNA1*) or *Not*I and *Bam*HI (for *EPHA2*). 18S RNA was used as a control. The probes were labeled with ^{32}P-dCTP using a random primed DNA labeling kit (Takara, Otsu, Japan). Hybridization was carried out at 42°C for 10 h. The hybridized membranes were washed three times at 42°C for 10 min and three times at 65°C for 30 min in 0.1% sodium dodecylsulfate (SDS) and 0.1× standard saline citrate. For normalization of signal intensity, the membranes were stripped and then rehybridized with an 18S probe. The autoradiographic densities were measured using a bio-imaging analyzer (BAS-1000; Fuji Film).

Protein extraction from tissue. Proteins were extracted from gastric cancer tissue and from non-tumor gastric tissue, dissected, and identified microscopically. They were powdered and homogenized in TXB (10 m*M* Tris [pH 7.6], 150 m*M* NaCl, 5 m*M* ethylenediamine tetra-acetic acid [EDTA], 10% glycerol, 1 m*M* Na_3VO_4, 1% TritonX-100, aprotinin [10 μg/mL], leupeptin [10 μg/mL], and phenyl-methyl-sulfonyl-fluoride [PMSF; 20 μg/mL]). After keeping on ice for 30 min, the proteins were centrifuged twice at 18 000 × *g* for 30 min. These lysates were then used for western blotting.

EPH receptor stimulation, immunoprecipitation, and western blots. A soluble form of ephrinA1-Fc, which is a mouse ephrinA1-human IgG1 Fc chimeric protein, was purchased from R&D Systems (Minneapolis, MN, USA). Human IgG1 Fc protein was used as a control (R&D Systems). The proteins were clustered using antihuman IgG, Fc_γ (Jackson Immuno Research Laboratories, West Grove, PA, USA) in RPMI with 0.5% FBS. The cells were incubated for 1 day, and then fed with a medium containing the clustered chimeric protein at 37°C. At the indicated times after stimulation, cells were harvested in lysis buffer (50 m*M* HEPES, 150 m*M* NaCl, 10% glycerol, 1% Triton X-100, 1.5 m*M* $MgCl_2$, 1 m*M* ethyleneglycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetra-acetic acid, 10 m*M* $Na_4P_2O_7$, 100 m*M* NaF, 1 m*M* Na_3VO_4) in the presence of protease inhibitors (aprotinin [10 μg/mL], leupeptin [10 μg/mL], and PMSF [20 μg/mL]). To pull down phosphorylated EPHA2, the cell lysates were incubated with anti-EPHA2 antibody (clone D7; Upstate Biotechnology, Lake Placid, NY, USA) followed by precleaning and immunoprecipitation with protein G sepharose (Amersham Biosciences). Immunoprecipitated lysates were then separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes (Amersham Biosciences), and immunoblotted with antiphosphotyrosine antibody (Upstate Biotechnology). The total lysate was also loaded in parallel for comparison.

Immunohistochemistry. The formalin-fixed paraffin-embedded gastric cancer tissue next to the portions taken for RNA and protein analysis was immunostained with anti-EPHA2 (C-20, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's instructions. We gathered nine cases of gastric adenoma and immunostained these in the same manner.

Antibody specificity was demonstrated using an immunoabsorption test with 6 μg/mL of EPHA2(C-20) blocking peptide (Santa Cruz Biotechnology). All the immunostains in this study were accompanied by this absorption test. The specificity of this antibody has also been tested by carrying out western blotting with this antibody to the lysate of 293T cells that were transfected with a plasmid that encoded EPHA2.

Cell growth assay. A quantity of 1×10^5 cells of MKN74 and TMK1 was seeded into each well of a six-well tissue culture dish (Greiner Bio-one, Kremsmüster, Austria). The cells were replenished with a medium that contained clustered ephrinA1-Fc (4 μg/mL) or clustered Fc every 24 h. After 3 days of incubation at 37°C, cells were harvested in trypsin-EDTA solution (0.05% Trypsin, 0.53 m*M* EDTA-4 Na; Invitrogen, Carlsbad, CA, USA) and the number of cells was counted using a hemacytometer. The experiment was repeated at least three times. Statistical analysis was carried out using Student's *t*-test (Microsoft Excel, Seattle, WA, USA), with $P < 0.05$ defined as significant.

Results

Expression of EPHA2 and EFNA1 in gastric cancer tisseus, paired non-tumor tissues, and gastric cancer cell lines. The authors examined the expression of *EPHA2* and *EFNA1* RNA in cancerous and non-cancerous gastric tissue using semiquantitative RT-PCR analysis (Fig. 1A). *EPHA2* was more highly expressed in tumor tissues than in normal tissues in 27 of 49 cases (55%). *EFNA1* was also overexpressed in substantial subsets of tumor tissues relative to the normal counterparts, specifically in 28 of 49 cases (57%). When classified using histology, *EPHA2* overexpression

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was more frequently detected in poorly differentiated adenocarcinoma (14/24 [58%]) than in well-differentiated adenocarcinoma (11/22 [50%]). Overexpression of *EFNA1* was more frequently detected in well-differentiated (15/22 [68%]) than in poorly differentiated adenocarcinoma (14/24 [58%]). However, these trends were not statistically significant. Generally, simultaneous expression of *EPHA2* and *EFNA1* in individual cases was not noted. Rather, the expression pattern was somewhat reciprocal.

When the authors classified the expression of both genes based on the depth of tumor invasion, *EPHA2* overexpression was more prominent in tumors that invaded deeper than the proper muscle layer of the gastric wall than in those that were within the mucosal and submucosal layers (depth mp, ss, se, and si *vs* depth m and sm, $P = 0.041$; lower-case abbreviations used as given in the JCS).[12] Furthermore, when the advanced cases ($n = 43$) were categorized into four macroscopic types according to the JCS, more frequent overexpression of EPHA2 was noted in type 3 and 4 tumors. That is, overexpression was more common in tumors that were macroscopically infiltrating with undefined margins or infiltrating diffusely, than in type 1 and 2 tumors, which are polypoid or ulcerated tumors with sharply demarcated margins ($P = 0.029$).

As for lymphatic invasion, it is difficult to estimate when tumor cells proliferate and are accompanied by severe stromal fibrosis. Therefore, the authors defined lymphatic invasion as 'mild' (corresponding to ly0 and ly1 in the JCS) or 'severe' (corresponding to ly2 and ly3 in the JCS). In cases with mild lymph vessel invasion, *EPHA2* was more frequently overexpressed than in cases with severe invasion, although this difference was not statistically significant ($P = 0.14$). No other significant correlations were detected when compared with age, lymph node involvement, or vessel invasion. A summary of the correlation between *EPHA2* and *EFNA1* expression and clinicopathologic characteristics is shown in Table 1.

The authors monitored EPHA2 protein expression in the gastric cancer tissues using immunohistochemistry and western blotting (Fig. 2). EPHA2 overexpression in cancer tissue compared with non-tumor tissue was confirmed in those cases in which EPHA2 overexpression was demonstrated using RT-PCR (Fig. 1A, case 2). In this case, EPHA2 was detected in a cancerous portion (Fig. 2A). The EPHA2 staining in the cancerous portion disappeared when absorption with EPHA2-blocking peptide, supplied by Santa Cruz, was carried out (Fig. 2B). Western blotting using this antibody to transfected cells further verified the specificity of the antibody (Fig. 2C).

The authors noted that RT-PCR data in some cases indicated considerable EPHA2 expression in non-tumorous gastric tissue. Immunohistochemical analysis of such cases demonstrated that EPHA2 immunoreactivity was detected not only in cancerous portions, but also in non-tumorous mucosa, mostly in that with intestinal metaplasia (Fig. 2D). Additionally, of the nine cases of adenomas that were investigated, three cases showed immunoreactivity to EPHA2, while the others did not show any (Fig. 2E,F).

In the cases that had EPHA2 overexpression in cancerous tissue, the authors could confirm EPHA2 overexpression using western blotting (Fig. 2G). Together, these data indicate that a substantial portion of gastric cancer, particularly infiltrative advanced cancer, is characterized by EPHA2 overexpression.

EPHA2 expression and phosphorylation by ephrinA1-Fc stimulation in gastric cancer cell lines. The *EPHA2* and *EFNA1* expression profiles in four gastric cancer cell lines were examined using RT-PCR. *EPHA2* was clearly detected in three gastric cancer cell lines (AGS, KATO3 and MKN74), but the band in TMK1 was fainter than that in the other three cell lines (Fig. 1B). In contrast, *EFNA1* seemed to be more abundantly expressed in TMK1 than in the other three cell lines (Fig. 1B). These data were also confirmed using northern blotting (Fig. 3). The

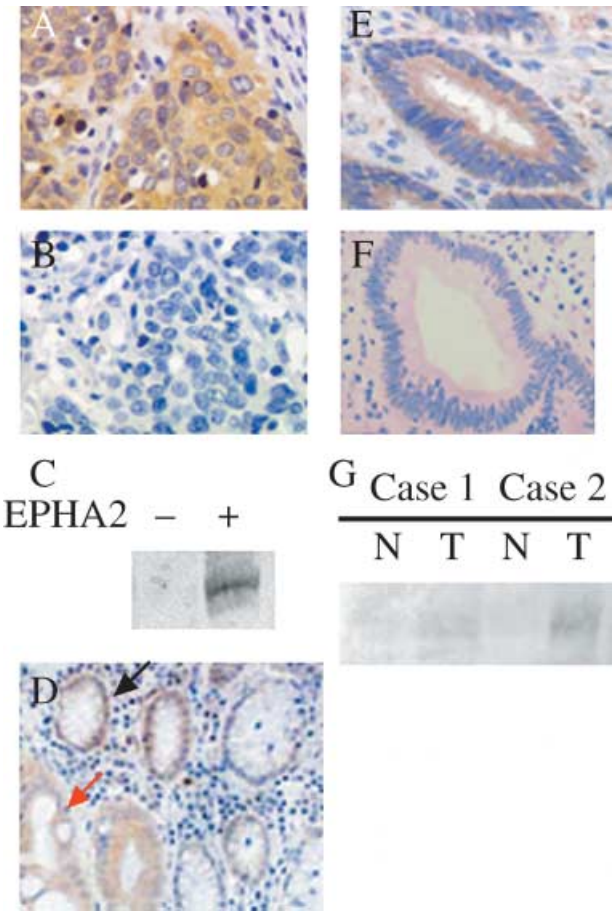

Fig. 2. Immunohistochemistry of EPHA2 in primary gastric cancer tissue. (A) EPHA2 was detected in cytoplasm and cell membrane of tumor cells. ×400. (B) The adjacent section after absorption with EPHA2 blocking peptide. ×400. (C) Western blotting using anti-EPHA2 antibody to cell lysates of 293T transfected with a mock (–) and an EPHA2 encoding plasmid (+). (D) Normal (black arrow) and intestinal metaplasia (red arrow) portion. ×400. (E,F) Gastric adenomas with (E) and without (F) EPHA2 immunoreactivity. (G) Western blotting of the primary tissues to anti-EPHA2 antibody. Case 1 and 2 are the same cases 1 and 2 shown in Fig. 1A. The lysates were from N (non-tumorous gastric tissue) and T (gastric cancer tissue).

authors believe the prevalence of *EPHA2* expression in gastric cancer cell lines (three of four) is consistent with that in primary gastric tumor tissue described above. Using MKN74, in which *EPHA2* expression is prominent, the effects of the soluble ligand, EFNA1, on the EPHA2 expression level and on phosphorylation were investigated. Interestingly, and as expected from the authors' previous data,[14] when MKN74 cells were stimulated by clustered ephrinA1-Fc chimeric protein, the EPHA2 phosphorylation increased and the EPHA2 protein degraded (Fig. 4). Both phenomena were dose- and time-dependent. The same results were also obtained for other *EPHA2*-expressing gastric cancer cell lines (AGS and KATO3; Fig. 4C). TMK1, in which *EPHA2* was faintly expressed, showed no change in expression and phosphorylation level as a result of ephrinA1-Fc stimulation.

EFNA1–EPHA2 signals inhibit cell growth. The authors further tested the effect of the ligand on anchorage-dependent growth of *EPHA2*-expressing gastric cancer cell lines. As shown in Figure 5, the growth of MKN74 was retarded by stimulation of soluble ephrinA1-Fc every 3 days, compared with cells treated with the control Fc protein ($P < 0.05$, using Student's *t*-test; Fig. 5). In contrast, in TMK1, which very weakly expresses EPHA2, cell growth was not changed by ephrinA1-Fc ($P = 0.53$; Fig. 5).

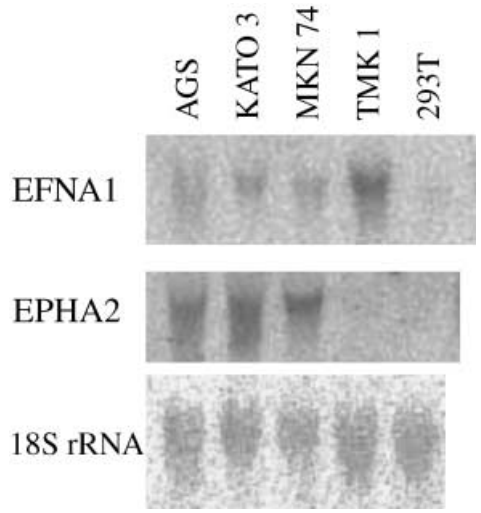

Fig. 3. EphrinA1 (EFNA1) and EPHA2 expression by northern blotting. (A) EFNA1 is expressed in all gastric cancer cell lines, particularly TMK1. (B) EPHA2 is clearly detected in AGS, KATO3, and MKN74, but very weakly in TMK1. These data were quantitatively consistent with those obtained using semiquantitative reverse transcription-polymerase chain reaction, shown in Figure 1. 293T is shown as a negative control.

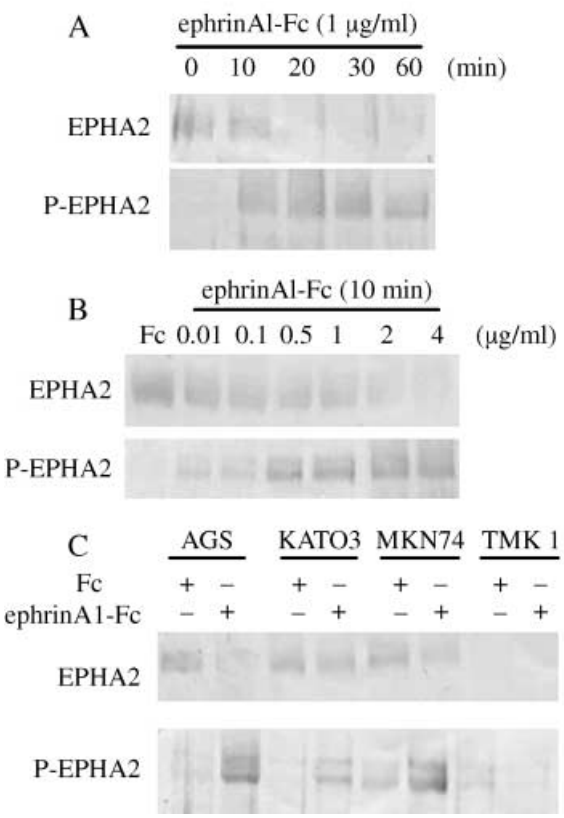

Fig. 4. Phosphorylation and degradation of EPHA2 stimulated by ephrinA1-Fc in MKN74 cells and other gastric cell lines. (A,B) MKN74 cells were stimulated by ephrinA1-Fc for the indicated periods (A) and at the noted concentrations (B). Total cell lysates were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with anti-EPHA2 antibody (upper panels). The cell lysates were immunoblotted with antiphosphotyrosine antibody after immunoprecipitation with anti EPHA2 and separated using SDS–PAGE (lower panels). (C) EPHA2 expression after EFNA1 stimulation in the other gastric cancer cell lines. AGS and KATO3 also showed an increase in EPHA2 phosphorylation and degradation of EPHA2 by ephrinA1-Fc.

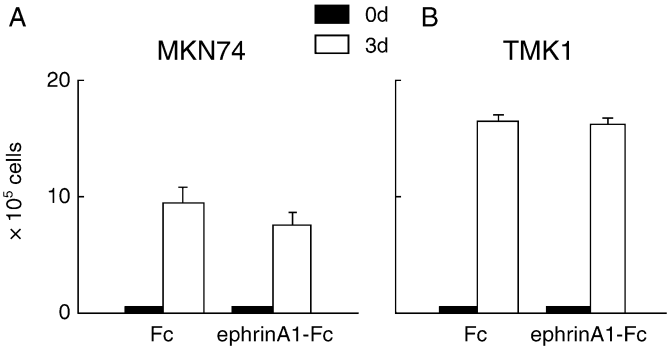

Fig. 5. EphrinA1-Fc inhibited growth of cells expressing EPHA2, but not cells that did not express EPHA2. (A) 1×10^5 of MKN74 or (B) TMK1 were seeded on a six-well dish and, after 24 h, fed with cell medium containing ephrinA1-Fc. The medium was changed every 24 h for 3 days, and the cell numbers were counted using a hemacytometer daily. EFNA1 stimulation inhibited MKN74 cell growth. The bars show means ± SE of cell numbers (three or more experiments).

Discussion

Receptor tyrosine kinases and their ligands play a critical role in regulation of cellular survival, proliferation, and differentiation.[15–17] *EPH* and *EFN* overexpression have been documented in many types of tumors, including gastrointestinal carcinoma. The authors have previously reported that *EPHB2* and *EFNB1* are overexpressed in gastric cancer.[18,19] With regard to *EPHA2*, a well-documented epithelial type EPH kinase, several studies have shown *EPHA2* overexpression in breast cancer,[8] advanced melanoma,[20] non-small-cell lung carcinoma,[20,21] prostate cancer,[23–25] renal cell carcinoma,[26] esophageal cancer,[9] and colorectal cancer. [10,13,27] Furthermore, as the authors have previously reported, *EPHA2* overexpression sometimes confers an angiogenic phenotype to colon tumors.[13]

The expression of *EFNA1* and *EPHA2* in 49 primary gastric cancer tissues and corresponding non-cancerous mucosa was examined in the present report. The authors found preferential *EPHA2* overexpression in advanced gastric cancer and, interestingly, in tumors with diffuse infiltrating margins with the surrounding tissue (macroscopic types 3 and 4).

Previous *in vitro* studies of breast cancer, pancreatic cancer, and malignant melanoma have shown that overexpression of *EPHA2* promotes malignant features of tumor cells, such as aggressiveness of cell growth, cellular invasiveness[28] and migration. EFNA1 or EPHA2 antibodies have been shown to negatively regulate EPHA2 expression and migration in human and rodent systems.[8,29–31] In light of these previous reports, the preferential overexpression in infiltrative and advanced gastric cancers reported here verifies the role of these molecules in human gastric carcinogenesis, in a physiological context. Overexpression of *EPHA2* gives gastric cancer more infiltrative characteristics.

There were some cases in which EPHA2 was detected in non-tumor tissue. As far as additional immunohistochemical analysis was extended to the premalignant gastric mucosae, EPHA2 was detectable in some, but not in all of these background mucosae (normal and intestinal metaplasia), as shown in Fig. 2D. It appears that the portions that were randomly selected and analyzed using RT-PCR, in some cases showed EPHA2 expression in the non-tumorous tissue. The authors also investigated gastric adenomas and found modest immunoreactivity in some (Fig. 2E,F). Generally, the immunoreactivity in the non-cancerous epithelial portion was less intense and more localized. However, there are a few reports of *EFNA1* expression profiles in human tumors. The authors also investigated the expression of *EFNA1*

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in gastric cancer, and some samples were found to overexpress *EFNA1*. However, the profile is not like that of *EPHA2*. For example, although not statistically significant, the *EFNA1* expression level seems to be lower in well-differentiated than in poorly differentiated adenocarcinoma, which is contrary to the situation for *EPHA2*. Moreover, in terms of the macroscopic type and the depth of tumor invasion in the gastric wall, the *EFNA1* expression profile was the reverse of that of *EPHA2*. The authors inferred that the following *in vitro* study would give some insight to explain this observation. Cell lines with high levels of EPHA2 (AGS, KATO3, and MKN74) exhibit lower levels of EFNA1, whereas those expressing high levels of EFNA1 (TMK1) exhibit lower levels of EPHA2. EPHA1 stimulation leads to EPHA2 degradation.

Further, the authors showed that EFNA1–EPHA2 participated in anchorage-dependent growth of gastric cancer cell lines. This seems to be related to the *EPHA2* status, because TMK1, a cell line that expresses a lower level of *EPHA2*, did not respond to EFNA1 stimulation.

Concerning the mechanism of *EPHA2* degradation involving ligand-mediated autophosphorylation and degradation, the authors have already reported a contribution from c-CBL, an adapter protein.[14,32] In the present study, when MKN74 was stimulated by ephrinA1-Fc, the binding of EPHA2 and c-CBL by co-immunoprecipitation was detected (data not shown). Thus, EFNA1-induced downregulation of EPHA2, described here in gastric cancer, also probably occurs through CBL–EPHA2 interaction.

In the present report the authors showed the *EPHA2* and *EFNA1* expression profiles in gastric cancer and gastric cancer cell lines. *EPHA2* expression, phosphorylation, and growth were regulated by EPHA1 in gastric cancer cell lines. Considering that several investigators are expecting EPHA2/EFNA1 to be a molecular target for therapy against pancreatic cancer,[28,29] it is plausible that this may also be the case with advanced gastric cancer.

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References

1 Bohme B, VandenBos T, Cerretti DP, Park LS, Holtrich U, Rubsamen-Waigmann H, Strebhardt K. Cell-cell adhesion mediated by binding of membrane-anchored ligand LERK- 2 to the EPH-related receptor human embryonal kinase 2 promotes tyrosine kinase activity. *J Biol Chem* 1996; **271**: 24747–52.
2 Mellitzer G, Xu Q, Wilkinson DG. Eph receptors and ephrins restrict cell intermingling and communication. *Nature* 1999; **400**: 77–81.
3 George SE, Simokat K, Hardin J, Chisholm AD. The VAB-1 Eph receptor tyrosine kinase functions in neural and epithelial morphogenesis in C. elegans. *Cell* 1998; **92**: 633–43.
4 Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R. Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev* 1999; **13**: 295–306.
5 Pandey A, Shao H, Marks RM, Polverini PJ, Dixit VM. Role of B61, the ligand for the Eck receptor tyrosine kinase, in TNF- alpha-induced angiogenesis. *Science* 1995; **268**: 567–9.
6 Hafner C, Schmitz G, Meyer S, Bataille F, Hau P, Langmann T, Dietmaier W, Landthaler M, Vogt T. Differential gene expression of Eph receptors and ephrins in benign human tissues and cancers. *Clin Chem* 2004; **50**: 490–9.
7 Easty DJ, Herlyn M, Bennett DC. Abnormal protein tyrosine kinase gene expression during melanoma progression and metastasis. *Int J Cancer* 1995; **60**: 129–36.
8 Zelinski DP, Zantek ND, Stewart JC, Irizarry AR, Kinch MS. EphA2 overexpression causes tumorigenesis of mammary epithelial cells. *Cancer Res* 2001; **61**: 2301–6.
9 Miyazaki T, Kato H, Fukuchi M, Nakajima M, Kuwano H. EphA2 overexpression correlates with poor prognosis in esophageal squamous cell carcinoma. *Int J Cancer* 2003; **103**: 657–63.
10 Ogawa K, Pasqualini R, Lindberg RA, Kain R, Freeman AL, Pasquale EB. The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. *Oncogene* 2000; **19**: 6043–52.
11 Kao HW, Chen HC, Wu CW, Lin WC. Tyrosine-kinase expression profiles in human gastric cancer cell lines and their modulations with retinoic acids. *Br J Cancer* 2003; **88**: 1058–64.
12 Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma – 2nd English edition. *Gastric Cancer* 1998; **1**: 10–24. Available from URL: http://www.jgca.jp/PDFfiles/JCGC-2E.PDF
13 Kataoka H, Igarashi H, Kanamori M, Ihara M, Wang JD, Wang YJ, Li ZY, Shimamura T, Kobayashi T, Maruyama K, Nakamura T, Arai H, Kajimura M, Hanai H, Tanaka M, Sugimura H. Correlation of EPHA2 overexpression with high microvessel count in human primary colorectal cancer. *Cancer Sci* 2004; **95**: 136–41.
14 Wang Y, Ota S, Kataoka H, Wang Y, Ota S, Kataoka H, Kanamori M, Li Z, Band H, Tanaka M, Sugimura H. Negative regulation of EphA2 receptor by Cbl. *Biochem Biophys Res Commun* 2002; **296**: 214–20.
15 Kullander K, Klein R. Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol* 2002; **3**: 475–86.
16 Flanagan JG, Vanderhaeghen P. The ephrins and Eph receptors in neural development. *Annu Rev Neurosci* 1998; **21**: 309–45.
17 Schlessinger J, Ullrich A. Growth factor signaling by receptor tyrosine kinases. *Neuron* 1992; **9**: 383–91.
18 Kataoka H, Tanaka M, Kanamori M, Yoshii S, Ihara M, Wang YJ, Song JP, Li ZY, Arai H, Otsuki Y, Kobayashi T, Konno H, Hanai H, Sugimura H. Expression profile of EFNB1, EFNB2, two ligands of EPHB2 in human gastric cancer. *J Cancer Res Clin Oncol* 2002; **128**: 343–8.
19 Kiyokawa E, Takai S, Tanaka M, Iwase T, Suzuki M, Xiang YY, Naito Y, Yamada K, Sugimura H, Kino I. Overexpression of ERK, an EPH family receptor protein tyrosine kinase, in various human tumors. *Cancer Res* 1994; **54**: 3645–50.
20 Easty DJ, Bennett DC. Protein tyrosine kinases in malignant melanoma. *Melanoma Res* 2000; **10**: 401–11.
21 Kinch MS, Moore MB, Harpole DH Jr. Predictive value of the EphA2 receptor tyrosine kinase in lung cancer recurrence and survival. *Clin Cancer Res* 2003; **9**: 613–18.
22 D'Amico TA, Aloia TA, Moore MB, Conlon DH, Herndon JE 2nd, Kinch MS, Harpole DH Jr. Predicting the sites of metastases from lung cancer using molecular biologic markers. *Ann Thorac Surg* 2001; **72**: 1144–8.
23 Walker-Daniels J, Coffman K, Azimi M, Rhim JS, Bostwick DG, Snyder P, Kerns BJ, Waters DJ, Kinch MS. Overexpression of the EphA2 tyrosine kinase in prostate cancer. *Prostate* 1999; **41**: 275–80.
24 Walker-Daniels J, Hess AR, Hendrix MJ, Kinch MS. Differential regulation of EphA2 in normal and malignant cells. *Am J Pathol* 2003; **162**: 1037–42.
25 Zeng G, Hu Z, Kinch MS, Pan CX, Flockhart DA, Kao C, Gardner TA, Zhang S, Li L, Baldridge LA, Koch MO, Ulbright TM, Eble JN, Cheng L. High-level expression of EphA2 receptor tyrosine kinase in prostatic intraepithelial neoplasia. *Am J Pathol* 2003; **163**: 2271–6.
26 Tatsumi T, Herrem CJ, Olson WC, Tatsumi T, Herrem CJ, Olson WC, Finke JH, Bukowski RM, Kinch MS, Ranieri E, Storkus WJ. Disease stage variation in CD4+ and CD8+ T-cell reactivity to the receptor tyrosine kinase EphA2 in patients with renal cell carcinoma. *Cancer Res* 2003; **63**: 4481–9.
27 Saito T, Masuda N, Miyazaki T, Saito T, Masuda N, Miyazaki T, Kanoh K, Suzuki H, Shimura T, Asao T, Kuwano H. Expression of EphA2 and E-cadherin in colorectal cancer: correlation with cancer metastasis. *Oncol Rep* 2004; **11**: 605–11.
28 Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. EphA2: a determinant of malignant cellular behavior and a potential therapeutic target in pancreatic adenocarcinoma. *Oncogene* 2004; **23**: 1448–56.
29 Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. Ligation of EphA2 by Ephrin A1-Fc inhibits pancreatic adenocarcinoma cellular invasiveness. *Biochem Biophys Res Commun* 2004; **320**: 1096–102.
30 Miao H, Wei BR, Peehl DM, Li Q, Alexandrou T, Schelling JR, Rhim JS, Sedor JR, Burnett E, Wang B. Activation of EphA receptor tyrosine kinase inhibits the Ras/MAPK pathway. *Nat Cell Biol* 2001; **3**: 527–30.
31 Carles-Kinch K, Kilpatrick KE, Stewart JC, Kinch MS. Antibody targeting of the EphA2 tyrosine kinase inhibits malignant cell behavior. *Cancer Res* 2002; **62**: 2840–7.
32 Walker-Daniels J, Riese DJ, 2nd, Kinch MS. c-Cbl-dependent EphA2 protein degradation is induced by ligand binding. *Mol Cancer Res* 2002; **1**: 79–87.