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Original Article

EPHB2 expression is associated with intestinal phenotype of gastric cancer and indicates better prognosis by suppressing gastric cancer migration

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Abstract: The protein tyrosine kinase Ephrin type-B receptor 2 (EPHB2) belongs to one of the intestinal stem cell signature genes and plays a crucial role in maintaining the crypt-villous axis. Herein, we aimed to investigate the expression of EPHB2 during gastric carcinogenesis and evaluated its prognostic and functional significance in gastric cancer (GC). EPHB2 expression was upregulated in intestinal metaplasia and GCs compared to normal antral and fundic glands. *EPHB2* mRNA levels were strongly correlated with the intestinal stem cell markers *OLFM4*, *LGR5*, and EPHB3. Notably, EPHB2 expression was significantly correlated with CDX2 expression, and *in vitro* studies demonstrated that CDX2 expression increased both EPHB2 transcription and protein levels. In a large cohort of GC patients, EPHB2 positivity was observed in 39% of 704 GCs and was negatively correlated with tumor differentiation, lymphovascular invasion, and tumor-node-metastasis stages. Notably, EPHB2 positivity was associated with better overall survival, and it was an independent prognostic marker in intestinal-type GCs. Overexpression of EPHB2 in GC cell lines, MKN-28 and MKN-74, reduced migration activity by suppressing phosphorylation of focal adhesion kinase, whereas no significant difference was observed in proliferation rates. Thus, we suggest that EPHB2 acts as a tumor suppressor in GCs and can be a prognostic marker in intestinal-type GCs.

Keywords: EPHB2, gastric cancer, immunohistochemistry, prognosis

Introduction

Ephrin (Eph) receptors represent the largest family of receptor tyrosine kinases, comprising two subclasses based on their affinities for each other and on sequence conservation, EphA and EphB [1-3]. They are located on the cell surface and transduce signals in a bidirectional manner when they bind with their ligands, ephrins A and B [1]. The interactions of Eph receptors with ligands contribute to diverse developmental processes by controlling cell sorting and migration [1, 2]. In particular, EPHB2 expression is most prominent in the intestinal epithelium and plays an important role in maintaining the correct positioning of the proliferative compartment in the crypt-villous axis [4]. EPHB2 is a direct transcriptional target of the Wnt/β-catenin pathway, which is consistent with the findings that EPHB2 positivity is predominantly seen in gastrointestinal (GI) tumors in which abnormally enhanced Wnt signaling by

mutations in *APC* or *β-catenin* genes is involved in the development of GI cancers [5]. It has been shown that EPHB2 promotes cell proliferation in the intestinal epithelium, while functioning as a tumor suppressor by controlling cell migration and inhibiting invasive growth in a kinase-independent manner [6].

The prognostic value of EPHB2 has been extensively investigated in colorectal cancer (CRC), and most studies have demonstrated that EPHB2 plays a tumor-suppressive role [7] and is associated with a better prognosis in CRC patients [5, 7-11]. However, a couple of studies have reported conflicting results in other cancer types such as cervical cancer [12], breast cancer [13], and glioblastoma [14], in which EPHB2 is involved in cancer proliferation and progression, indicating an oncogenic role. For gastric cancers, only two studies have analyzed the impact of EPHB2 expression on clinical outcomes. Yu *et al.* reported that the loss of EPHB2 expression is associated with lymph node metastasis and poor survival rates in 337 GC patients [15]. In contrast, Yin *et al.* recently showed that EPHB2 expression is associated with poor overall survival and promotes the migration and invasion abilities of GC cells [16]. Therefore, it remains controversial whether EPHB2 exerts a tumor-suppressive or oncogenic role in GCs. In this study, we aimed to investigate the expression of EPHB2 in GCs and clarify its prognostic significance in a large cohort of GC patients.

Materials and methods

Participants

A total of 704 formalin-fixed and paraffin-embedded (FFPE) GCs were obtained from patients who underwent curative gastrectomy at Seoul National University Hospital (SNUH), Seoul, Korea, between 2004 and 2005 [17]. Clinicopathological data such as patient age, sex, histological type, evidence of lymphatic, venous, and perineural invasion, and tumor-node-metastasis (TNM) stages (7th edition) were obtained by thoroughly reviewing the medical and pathological records. In addition, 37 paired, fresh-frozen GC tissues and matched non-cancerous tissues were provided by the Jeju National University Hospital (JNUH) Biobank, a member of the National Biobank of Korea, for which informed consent was obtained from all participants. For validation studies, gene profiles and clinical information were obtained from two independent GC cohorts; 434 GC patients from the GSE84437 dataset (https://www.ncbi.nlm.nih.gov/geo/) and 67 GC patients from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). For validation studies, gene profiles and clinical information were obtained from two independent GC cohorts; 434 GC patients from the GSE84437 dataset (https://www.ncbi.nlm.nih.gov/geo/) and 67 GC patients from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). This study was approved by the Institutional Review Board of SNUH (H-1209-037-424) and JNUH (IRB No. 2016-10-001), and the institutional review board confirmed that informed consent was waived due to the retrospective nature of the study. All procedures were performed in accordance with the Helsinki Declaration of 1964 and later versions.

Tissue microarray construction

Fourteen TMAs containing 704 GCs from the Seoul National University Hospital were generated as previously described [17]. In addition, four cases of normal gastric tissue and intestinal metaplasia were included in the TMAs. Briefly, through histologic examination, the representative tumor area in which tumor cells make up at least 70% of the cell population was marked in each case. Tumor cores (2 mm in diameter) were extracted from individual FFPE gastric tumors (donor blocks) and placed in a new recipient paraffin block (tissue array block) using a trephine apparatus (SuperBioChips Laboratories, Seoul, Korea).

Immunohistochemistry interpretation

Immunohistochemistry was performed on TMA sections using a BOND-MAX automated immunostainer and a Bond Polymer Refine Detection kit (Leica Microsystems, Wetzlar, Germany) according to the manufacturer's instructions [18]. The primary antibodies used were anti-EPHB2 (R&D Systems, Minneapolis, MN, USA; 1:700) and CDX2 (BioGenex, CA, USA, 1:300). The positivity criteria for EPHB2 were based on previous studies [8, 11]. The expression of EPHB2 was determined by evaluating the tumor cell membranes. For each tumor, the intensity and percentage of tumor cells expressing

EPHB2 were assessed. Histo-scores (H-scores) were calculated by multiplying the intensity (0 = negative; 1 = weak; 2 = moderate; 3 = strong) and the percentage of positive tumor cells (range = 0-100), ranging from 0 to 300. For statistical analyses, we used a cutoff of 20 based on the distribution of the H-scores (median value: 40). GCs with an H-score of 20 or lower were considered negative, while cases with H-scores higher than 20 were considered positive. CDX2 was interpreted as positive when more than 10% of the tumor cell nuclei were strongly stained.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from the 37 paired fresh-frozen GCs and corresponding non-cancerous gastric tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1-2 μg) was subjected to reverse transcription using the GoScript reverse transcription system (Promega, Madison, Wisconsin, USA). Quantitative RT-PCR reactions were performed using Premix EX Taq (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Cycling conditions were as follows: initial denaturation for 30 s at 95°C, followed by 40 cycles of 95°C for 1 s and 60°C for 5 s in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the 7500 system SDS (Ver. 1.4) software (Applied Biosystems). The TaqMan gene expression assay was performed as follows: Hs00362096_m1 (EPHB2), Hs00173664_m1 (LGR5), Hs00197437_m1 (OLFM4), Hs01009250-m1 (PROM1/CD133), Hs01075864_m1 (CD44), Hs00946916_m1 (ALDH1A1), and Hs0275899_g1 (GAPDH). GAPDH served as an endogenous control.

Gastric cancer cell lines

Fifteen human gastric carcinoma cell lines (SNU-1, SNU-16, SNU-216, SNU-601, SNU-620, SNU-638, SNU-668, SNU-719, MKN-1, MKN-28, MKN-45, MKN-74, AGS, Kato3, and NCI-N87) were obtained from the Korean Cell Line Bank (Seoul, Korea). Cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics (penicillin G and streptomycin) in a humidified incubator containing 5% CO_2.

Transfection of CDX2, EPHB2, and siRNA

Full-length cDNA encoding CDX2 (pCMV6-CDX2), EPHB2 (pCMV6-EPHB2), and pCMV6-EGFP were purchased from Origene (Rockville, MD, USA). The siRNA pool targeting EPHB2 and the non-targeting siRNA pool were purchased from Dharmacon (Lafayette, CO, USA). Cells (1×10^6 cells/well) were seeded in a 6-well plate and transfected with 5 μg of cDNA or EPHB2 siRNA using the Invitrogen Neon transfection system (Thermo Fisher Scientific, USA). The pCMV6-EGFP vector and non-targeting siRNA pool were used as controls. Twenty-four hours after transfection, the cells were subjected to proliferation and migration assays. RNA was extracted for real-time PCR, and proteins were extracted for western blotting. All experiments were independently performed at least twice.

Western blot analysis

Proteins were extracted using lysis buffer (iNtRON Biotechnology, Seongnam, Korea) and quantitated using a BCA protein assay kit (Pierce, Rockford, IL, USA). Cell lysates were run on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA). The membrane was blocked with 5% non-fat dry milk in PBS-Tween-20 (0.1%, *v/v*) for 1 h and then incubated with specific primary antibodies: CDX2 (BioGenex), EPHB2 (R&D systems), HSP90 (Origene), GAPDH (Origene), FAK (Cell signaling, Danvers, MA), and phospho-FAK (Tyr925) (Cell Signaling Technology) overnight at 4°C, washed with TBS containing 0.1% Tween-20, the membrane was incubated for 1 h with secondary antibodies. The Alliance-Mini HD9 chemiluminescence documentation system (UVItec Cambridge, UK) was used to visualize the target proteins.

Proliferation assay

For the proliferation assay, 5×10^3 cells/well were counted using LUNA-II (Logos Biosystems, Gyeonggi-do, Korea) and were seeded in the wells of a 96-well plate at 37°C. After adding 10 μl of Cell Counting Kit-8 reagent (Dojindo, Kumamoto, Japan) to each well and incubating for 1 h, the optical density was measured at 450 nm using an automatic microplate reader (Thermo Labsystems, Rockford, IL, USA).

Experiments were performed in triplicate and repeated twice independently.

Wound healing assay

Cells were transfected with the control or EPHB2 plasmid DNA and were cultured in a SPL Scar Block (SPL Life Sciences, Seongnam, Korea) that is composed of 500 μm-thick walls to generate empty (cell-free) gaps. The block was removed from the plate when the cells were confluent, and the culture medium was added. Cellular migration was monitored and photographed at 0 and 72 h. Experiments were performed in duplicate and repeated twice independently.

Statistical analysis

Statistical analyses were performed using the PASW 18.0 statistical software program (IBM SPSS Statistics, Chicago, IL, USA) and Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA; https://www.graphpad.com/scientific-software/prism). Between-group comparisons were performed using Student's t-test or Tukey's multiple comparison test. Correlations between stem cell-related markers were evaluated using the Spearman correlation test. Survival curves were estimated using the Kaplan-Meier method, and the log-rank test was used to compare groups. The Cox proportional hazards model was used to compare hazard ratios in the multivariate analyses. Statistical significance was set at *P*-value <0.05.

Results

EPHB2 expression in gastric cancers and its correlation with stem cell-related markers

To determine the expression of EPHB2 and stem cell-related markers in GCs, real-time PCR was performed on a series of 37 pairs of fresh GC samples as well as on matched non-cancerous gastric tissues. Compared to normal mucosa, *EPHB2* mRNA levels were significantly higher in the majority of GC samples examined (89%, 33 out of 37 cases) (**Figure 1A**). The mean *EPHB2* expression level was higher in GCs (mean ± SD: 0.052±0.068) than in matched normal tissues (mean ± SD: 0.0069±0.0042); (P = 0.017) (**Figure 1B**). The mean expression levels of other stem cell-related markers are shown in Supplementary Figure 1. As *EPHB2* is enriched in the stem cells of the intestinal crypts, we examined whether there was any correlation between *EPHB2* and other intestinal stem cell (ISC) markers, including *OLFM4*, *LGR5*, and *EPHB3*. We found that *OLFM4* (r^2 = 0.55, P<0.0001) and *LGR5* (r^2 = 0.20, P = 0.007) were positively associated with *EPHB2* (**Figure 1C**). Additionally, we investigated the association of *EPHB2* with candidate cancer stem cell (CSC) markers that have been suggested in GCs, such as *CD133*, *CD44*, and *ALDH1A1*. However, none of them showed significant correlations with *EPHB2* expression (**Figure 1D**).

Close association of EPHB2 expression with intestinal differentiation

We examined the expression profile of EPHB2 in normal gastric mucosa and intestinal metaplasia. Immunohistochemical analysis of normal antral and fundic glands showed no EPHB2 expression, whereas EPHB2 expression appeared at the bottom of the glands in intestinal metaplasia (IM) with moderate staining intensity (**Figure 2A**). This is not surprising considering that EPHB2 belongs to a group of intestinal stem cell signature genes. To study whether the association between EPHB2 and intestinal differentiation remains in GCs, we investigated the correlation between *EPHB2* and *CDX2* expression in 37 GC samples. *CDX2* was positively correlated with *EPHB2* (r^2 = 0.31, P< 0.001) and *OLFM4* (r^2 = 0.18, p<0.01), but not with *LGR5* (r^2 = 0.09, P = 0.07) (**Figure 2B**). None of the CSC markers showed a significant correlation with *CDX2* (CD133: r^2<0.01, P = 0.90; *CD44*: r^2<0.01, P = 0.86, *ALDH1A1*: r^2<0.01, P = 0.68) (**Figure 2C**). Since CDX2 is a master transcription factor for intestinal differentiation, we determined that CDX2 may directly induce EPHB2 expression. We confirmed a positive association between EPHB2 and CDX2 expression in 15 gastric cancer cell lines (r^2 = 0.09, P = 0.07) (**Figure 3A**, **3B**). An *in vitro* assay demonstrated that CDX2 transfection led to upregulation of EPHB2 expression in MKN-28 and MKN-74 cells (**Figure 3C**, **3D**). These findings suggest that enhanced EPHB2 expression in GCs is closely associated with intestinal differentiation induced by CDX2.

Prognostic significance of EPHB2 in gastric cancer patients

IHC was performed on tissue microarrays containing a large group of patients with GC

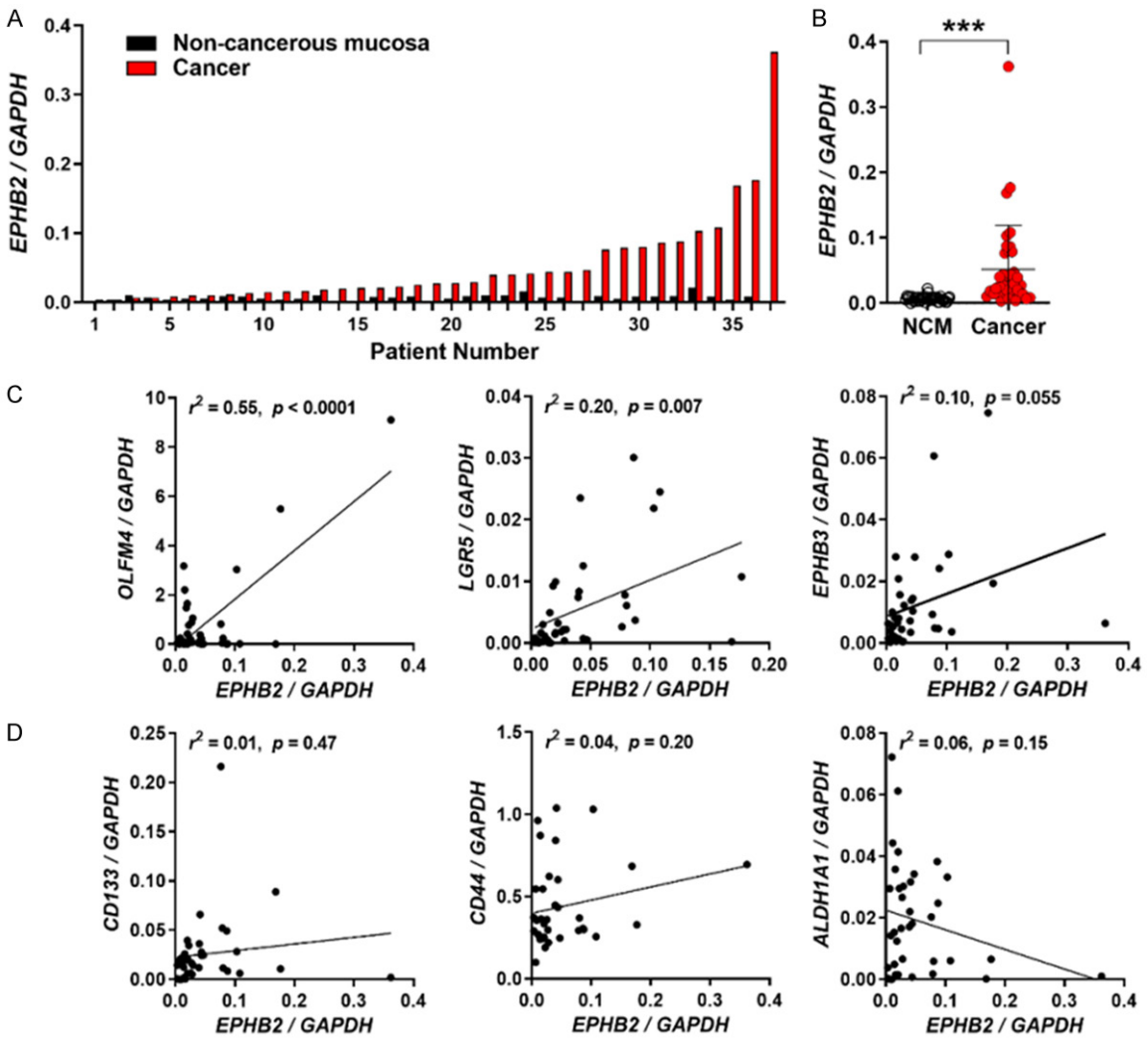

Figure 1. *EPHB2* mRNA expression in gastric cancers (GCs) and its correlation with intestinal stem cell (ISC) and cancer stem cell (CSC) markers. Real-time PCR analysis measured the expression levels of *EPHB2* and ISC (*OLFM4, LGR5,* and *EPHB3*) and CSC markers (*CD133, CD44*, and *ALDH1A1*) from 37 pairs of fresh-frozen GCs and matched non-cancerous mucosa (NCM). A, B. *EPHB2* mRNA expression was significantly higher in GCs than in NCM (n = 37). C, D. Scatter plots showing the correlations between *EPHB2* and ISC or CSC markers expression (n = 37). Data are presented as the mean ± SD. $^{***}P$<0.001.

(n = 704). Representative images of EPHB2-negative and EPHB2-positive GCs are shown in **Figure 4A**. The association between EPHB2 positivity and various clinicopathological parameters is summarized in **Table 1**. EPHB2 positivity was significantly higher in GCs with well-differentiated GCs than in poorly differentiated or signet-ring cell carcinomas (P<0.001) (**Table 2**). EPHB2-positive GCs had less lymphatic (P = 0.006) and venous (P = 0.013) invasion, and a lower TNM stage (P<0.001). In addition, EPHB2 positivity showed a strong positive correlation with CDX2 expression (P<0.001), which is consistent with the results of our RT-PCR and *in vitro* studies. EPHB2 was not correlated with age, sex, or Lauren classification.

Survival analysis demonstrated that EPHB2 positivity was significantly associated with better clinical outcomes (P<0.001) (**Figure 4B**). As intestinal- and diffuse-type GCs involve different molecular pathways, we separately analyzed the prognostic value of EPHB2 in intestinal, mixed, and diffuse type GCs. Notably, the prognostic value of EPHB2 remained significant in intestinal-type GCs (P<0.001), but not in mixed (P = 0.120) and diffuse-type GCs (P = 0.176). Additionally, the prognostic impact of

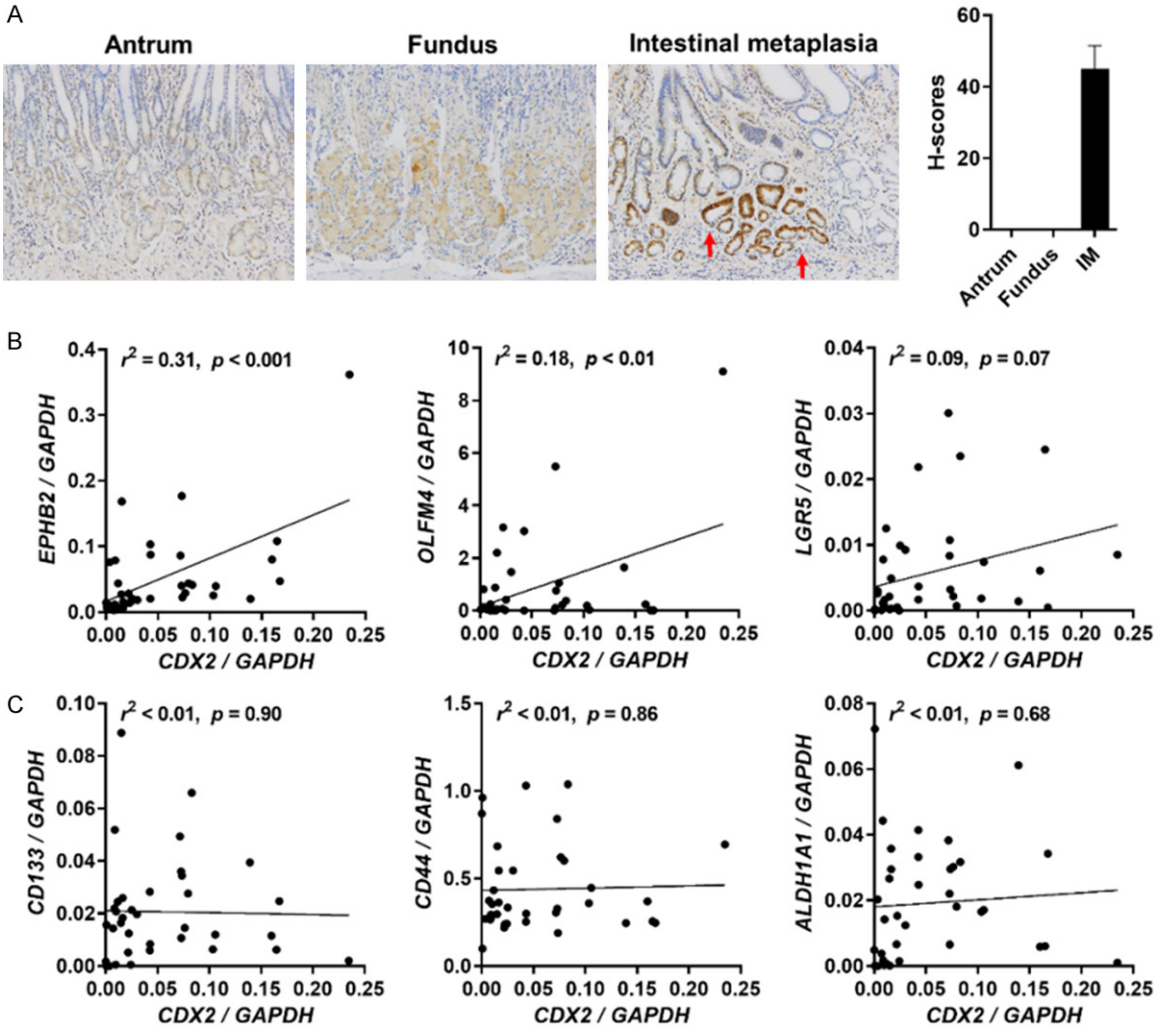

Figure 2. Correlation between EPHB2 expression and intestinal differentiation. A. Immunohistochemical analysis demonstrated increased EPHB2 expression in intestinal metaplasia (n = 4), and no EPHB2 expression was observed at the antral (n = 4) and fundic glands (n = 4). B. Scatter plots showing the correlations between *CDX2* and intestinal stem cell markers including EPHB2, OLFM4, and LGR5 in gastric cancers (n = 37). C. Scatter plots showing the correlations between *CDX2* and candidate cancer stem cell markers such as CD133, CD44, and ALDH1A1 in gastric cancers (n = 37). H-scores, histoscores. IM, intestinal metaplasia.

EPHB2 was significant in advanced GC (P = 0.006) and in CDX2-negative GCs (P = 0.002), but not in early GC (P = 0.397) and CDX2-positive GCs (P = 0.931) (**Figure 4C**, **4D**). When analyzing the prognostic significance of EPHB2 according to TNM stage, it was most apparent in stage II, although it did not reach statistical significance (Supplementary Figure 2). Multivariate analysis revealed that EPHB2 expression was not an independent prognostic factor in all cases (Supplementary Table 1). However, for intestinal-type GCs, EPHB2 was found to be an independent prognostic factor (HR: 0.579, P = 0.043) and tumor stage (HR: 3.795, P<0.001) (**Table 2**).

To validate the prognostic significance of EPHB2 expression, survival analysis was performed using two independent GC cohorts; GSE84437 and The Cancer Genome Atlas (TCGA) databases. In the GSE84437 GC cohort (n = 434), GC patients with high-EPHB2 expression showed significantly better overall survival (P = 0.042) (Supplementary Figure 3A). In the TCGA cohort, EPHB2 expression was positively associated with improved overall survival (P =

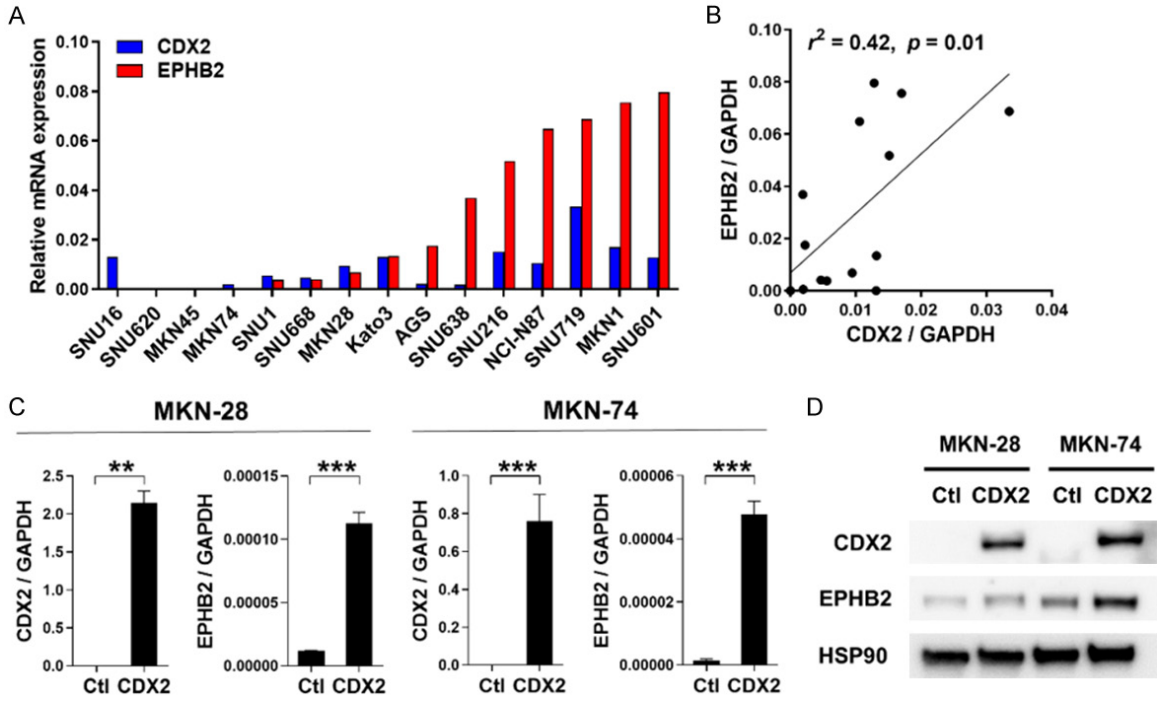

Figure 3. Effect of CDX2 on the expression of EPHB2 in gastric cancers (GCs). A. CDX2 and EPHB2 expression in 15 GC cell lines. B. A scatter plot showing the correlation of CDX2 with EPHB2 expression in GC cell lines (n = 15). C. Influence of CDX2 overexpression in MKN-28 and MKN-74 cells on the expression of EPHB2. D. Western blot for CDX2 and EPHB2 after transfection of CDX2-expressing plasmid and control vector. Ctl, control. **P<0.01, ***P<0.001.

0.004) (Supplementary Figure 3B) and disease-free survival (P<0.001) (Supplementary Figure 3C).

Effects of EPHB2 expression on the growth and migration of GC cells

To investigate the functional roles of EPHB2 in GCs, we screened 15 GC cell lines and selected MKN-28 and MKN-74 because of their low EPHB2 expression. We increased EPHB2 expression in two cell lines by transfecting EPHB2-expressing or control vectors, which was confirmed by immunoblot assay (**Figure 5A**). However, no significant difference in growth rates was observed between the cancer cells transfected with EPHB2 and those transfected with the control plasmid (**Figure 5B**). We also investigated the impact of EPHB2 expression on migration ability using a wound-healing assay. Notably, increased EPHB2 expression significantly suppressed GC cell migration (P<0.001) (**Figure 5C**). To evaluate whether EPHB2 expression affects the signaling molecules involved in cell motility, we examined the phosphorylation level of focal adhesion kinase (FAK), which plays a key role in tumor cell migration and is regulated by numerous stimuli [19]. FAK phosphorylation significantly decreased in EPHB2-transfected cells (**Figure 5D**), suggesting that EPHB2-induced suppression of migration capability is mediated by the downregulation of FAK signaling activity. To further confirm the EPHB2 effects on GC cell growth and migration, EPHB2 expression was suppressed in MKN-28 and MKN-45 cells by transfecting the siRNA pool targeting EPHB2. Immunoblot analysis showed that marked EPHB2 downregulation was observed in MKN-28 cells, in which phospho-FAK significantly increased. On the other hand, EPHB2 suppression was weak in MKN-45 cells and there was no significant change in phospho-FAK expression (**Figure 6A**). Enhanced growth (**Figure 6B**) and migratory activity (**Figure 6C**) were observed only in MKN-28 cells. Thus, these results suggest that EPHB2 may have suppressive roles in the proliferation and migration of GCs.

Discussion

In this study, we examined the expression of EPHB2 in a large cohort of GC patients and

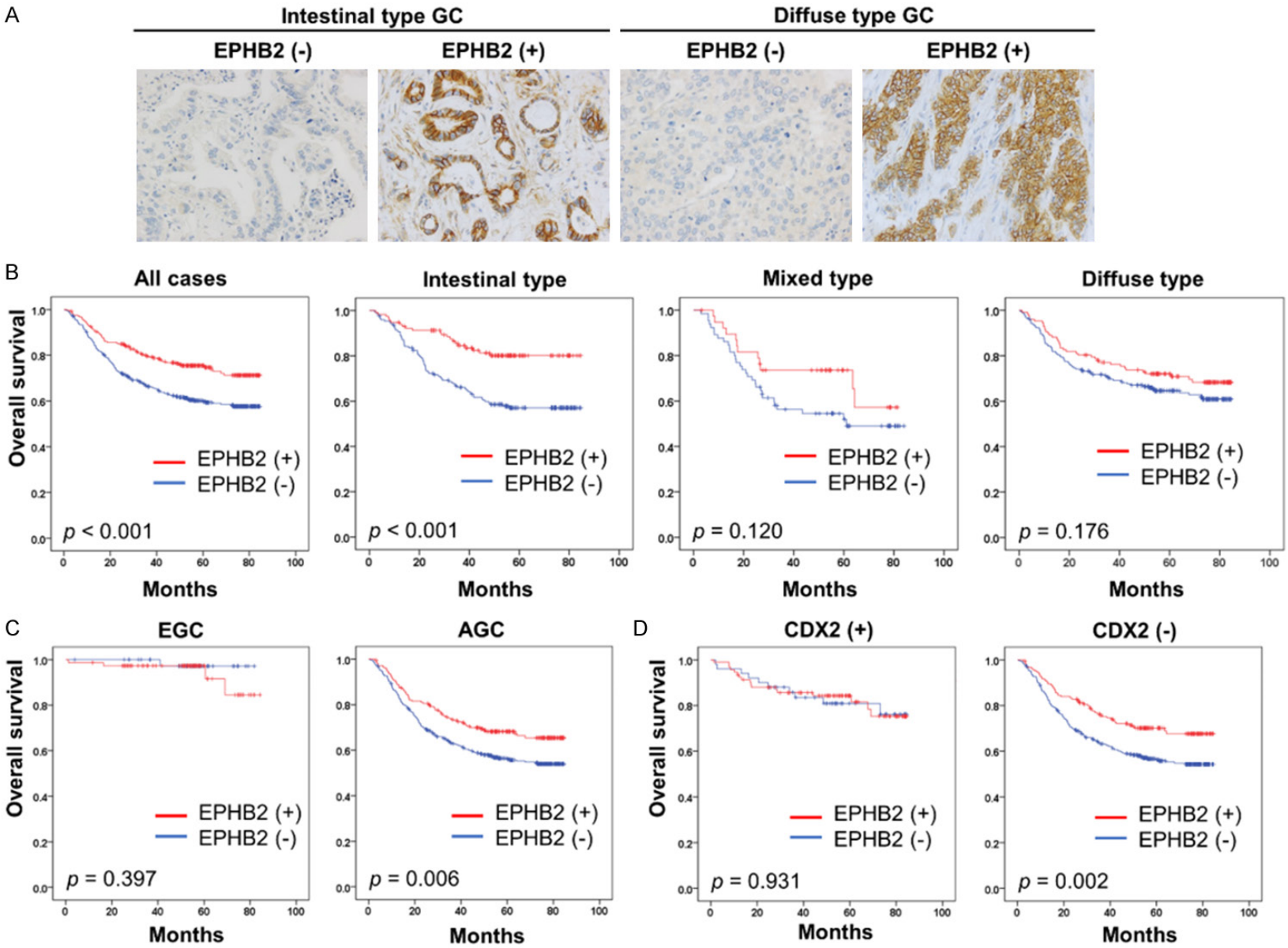

Figure 4. Prognostic significance of EPHB2 in gastric cancer (GC) patients. (A) Representative images of EPHB2 negativity and positivity in intestinal and diffuse type GCs. (B) Prognostic value of EPHB2 positivity in all cases (*n* = 733) or intestinal (*n* = 287), mixed (n = 104), and diffuse type (*n* = 337) GCs. (C) Overall survival of

GC patients with EPHB2 expression in early gastric cancers (EGCs, *n* = 117) and advanced gastric cancers (AGCs, *n* = 616) (C), and in CDX2-negative (*n* = 142) and CDX2-positive GCs (*n* = 562) (D).

Table 1. Association between the EPHB2 expression and the clinicopathological characteristics

Characteristics	Total (%)	EPHB2		*P*-value
		Negative (%)	Positive (%)	
Patients	704 (100)	431 (61)	273 (39)	
Age				
≥65	239 (34)	136 (57)	103 (43)	0.102†
<65	465 (66)	295 (63)	170 (37)	
Gender				
Female	222 (31)	137 (62)	85 (38)	0.868†
Male	482 (69)	294 (61)	188 (39)	
Lauren				
Intestinal	275 (39)	163 (59)	112 (41)	0.373#
Diffuse	329 (47)	204 (62)	125 (38)	
Mixed	96 (14)	60 (63)	36 (37)	
Undetermined	4 (1)	4 (100)	0 (0)	
Histological				
Well	147 (21)	69 (47)	78 (53)	<0.001#
Moderate	284 (40)	173 (62)	111 (38)	
Poor	173 (25)	120 (69)	53 (31)	
Signet ring cell	79 (11)	54 (68)	25 (32)	
Others	21 (3)	18 (86)	3 (14)	
Lymphatic invasion				
Negative	242 (34)	128 (53)	114 (47)	0.001†
Positive	462 (66)	303 (66)	159 (34)	
Venous invasion				
Negative	583 (83)	344 (60)	239 (40)	0.008†
Positive	121 (17)	87 (72)	34 (28)	
Perineural invasion				
Negative	306 (44)	163 (53)	143 (47)	<0.001†
Positive	398 (56)	268 (67)	130 (33)	
TNM_7th				
I	173 (25)	77 (45)	96 (55)	
II	199 (28)	123 (62)	76 (38)	<0.001#
III	253 (36)	173 (68)	80 (32)	
IV	79 (11)	58 (73)	21 (27)	
CDX2				
Negative	562 (80)	381 (68)	181 (32)	<0.001†
Positive	142 (20)	50 (35)	92 (65)	

†Fisher's exact test. #Pearson Chi-square. NM, tumor-node-metastasis.

determined its clinicopathological and prognostic significance. EPHB2 positivity was associated with well-differentiated histology and less aggressive behavior, such as lower lymphovascular and perineural invasion, and lower TNM stages. EPHB2 was closely associated with CDX2 levels, and its expression was upregulated by CDX2. More importantly, EPHB2 was found to be an independent prognostic marker for intestinal-type GCs.

Here, we observed that 39% of GCs examined were positive for EPHB2, which is comparable with the results of previous studies, ranging from 30% to 47% positivity in GCs [5, 15]. Interestingly, Lugli *et al*. showed that EPHB2 positivity was found in 100% of colon adenomas, and it declined to 33.3% in colon carcinomas [5]. Loss of EPHB2 expression is a strong indicator of poor overall survival in patients with colorectal cancer (CRC) [5, 8]. This inactivation of EPHB2 in colorectal cancers was suggested to be derived from frameshift mutations and promoter hypermethylation [20]. Several somatic mutations and biallelic inactivation of EPHB2 have also been detected in prostate cancer [21]. In addition, a nonsense mutation was identified as a genetic risk factor for prostate cancer development [22]. Frequent frameshift mutations in EPHB2 were detected in 41% of GCs with microsatellite instability (MSI), and it was suggested that this high frequency of EPHB2 mutations may confer gastric tumor cells a growth advantage [23]. On the other hand, Yu *et al.* and Song *et al*. have reported no frameshift mutation, but 21% and 62% of allelic loss in Chinese and Korean GC patients, respectively [15, 24]. Thus, it results in the genetic abnormalities of EPHB2 in GCs, indicating that more

Table 2. The results of multivariate analysis for survival rate for intestinal type gastric cancers

Variables	Category	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value[a]
Age	>65/<65	0.793 (0.505-1.244)	0.312		
Gender	Female/Male	0.926 (0.595-1.440)	0.732		
Location	Upper/Middle/Low/Whole	0.958 (0.704-1.305)	0.786		
Histology	WD/MD/PD/SRC	1.495 (0.999-2.238)	0.050	1.566 (0.931-2.636)	0.091
Lymphatic invasion	Positive/Negative	3.990 (2.291-6.950)	0.000	1.886 (0.996-3.571)	0.052
Venous invasion	Positive/Negative	3.690 (2.828-4.816)	0.000	1.334 (0.816-2.182)	0.250
Perineural invasion	Positive/Negative	4.042 (2.411-60776)	0.000	1.406 (0.819-2.13)	0.216
Tumor stage	IV/III/II/I	4.076 (3.081-5.392)	0.000	3.795 (2.647-5.441)	<0.001
CDX2	Positive/Negative	0.234 (0.102-0.537)	0.001	0.589 (0.247-1.403)	0.232
EPHB2	Positive/Negative	0.395 (0.242-0.644)	0.000	0.579 (0.340-0.984)	0.043

HR, Hazard ratio; CI, confidence interval. [a]Cox proportional hazard model.

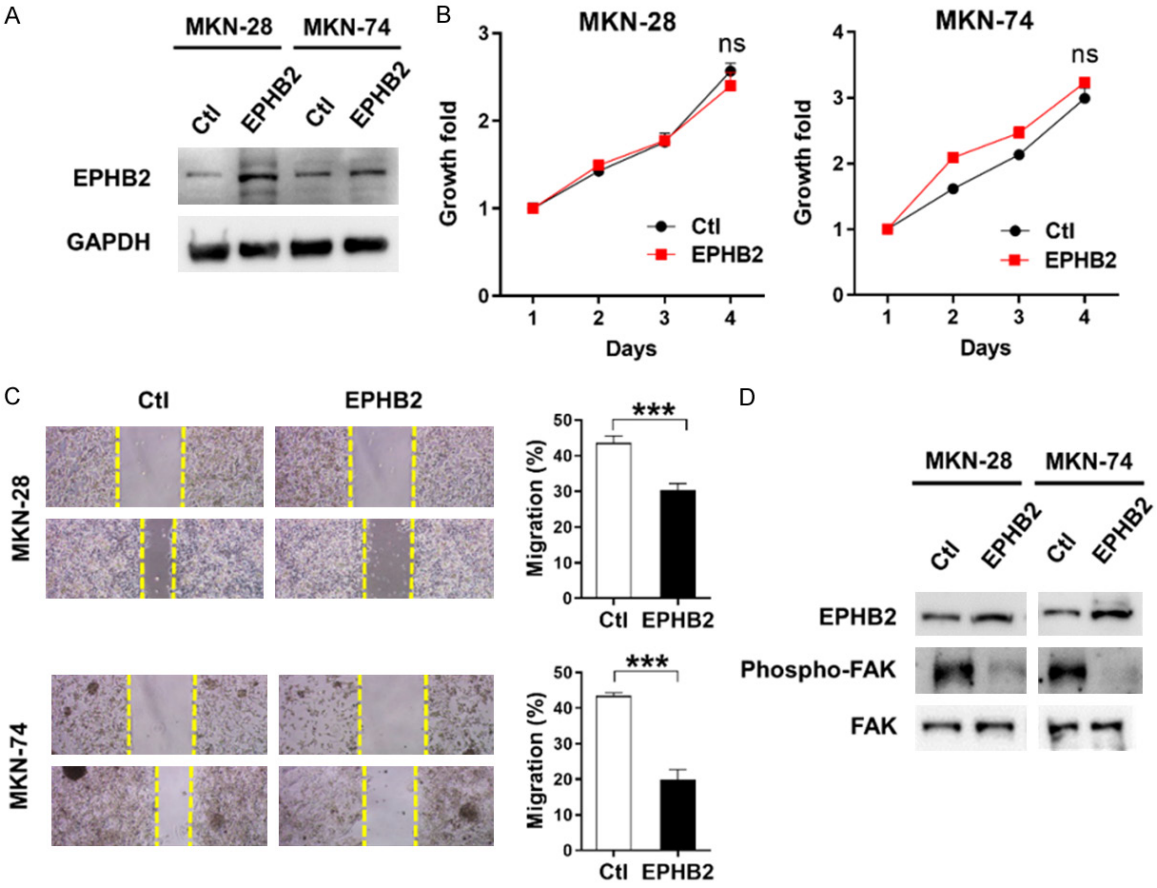

Figure 5. The effects of EPHB2 overexpression on gastric cancer (GC) cell growth and migration. A. EPHB2 expression was up-regulated by transfection with pCMV6-EPHB2 or PCMV6-EGFP in MKN28 and MKN74. Immunoblot analysis was performed using the antibodies indicated in the figure. B. Cell growth was determined using the Cell counting Kit-8 at the indicated times. C. The effect of EPHB2 up-regulation on the migration of MKN28 and MKN74 was evaluated by Wound healing assay. Cellular migration was photographed at 0 and 48 h. Yellow dashed lines indicate the approximate edge of the wound. Data are presented as the mean ± SD. D. Approximately, 24 hours after transfection with control plasmid or EPHB2 expressing-plasmid, immunoblot assay was performed using the antibodies indicated in the figure. Ctl, control; ns, not significant. ***P<0.001.

meticulous studies are required to reveal the precise molecular alterations in EPHB2 that are implicated in GC development and progression.

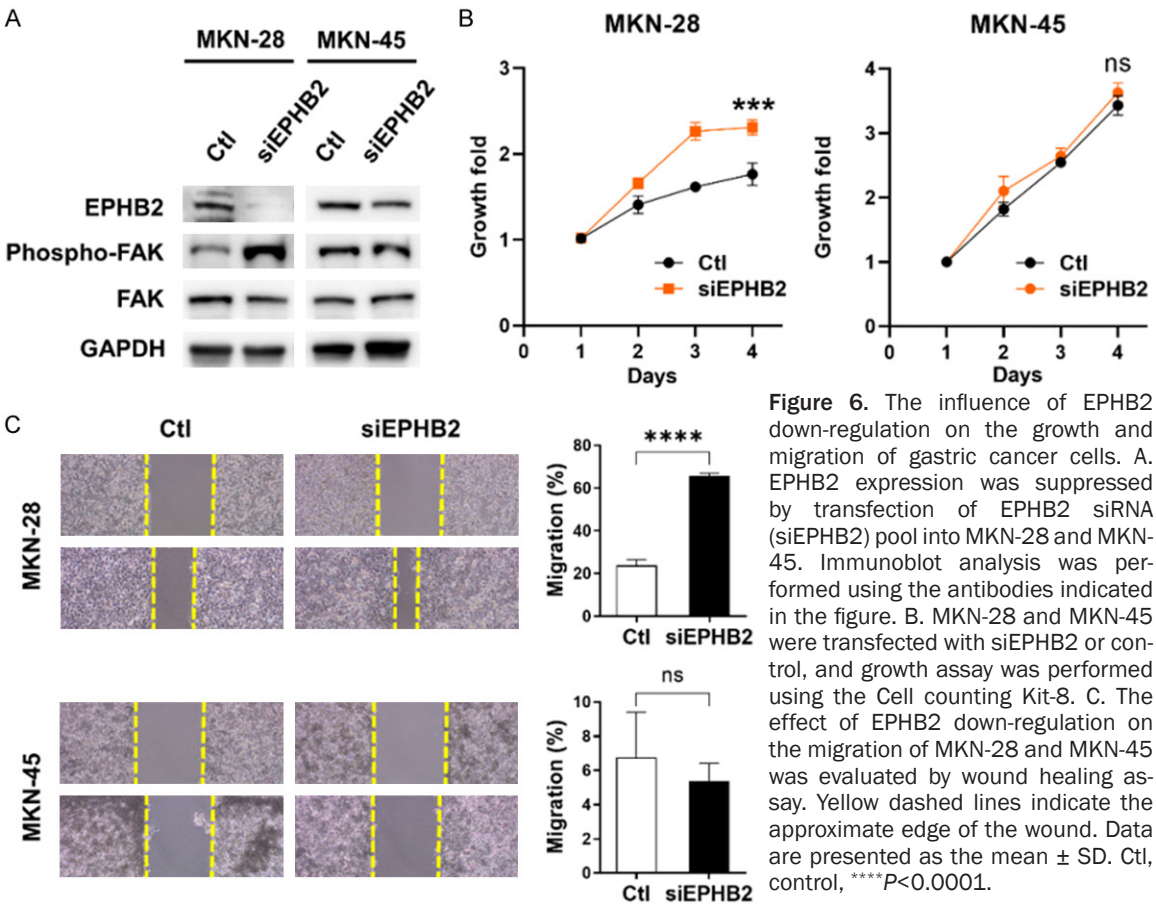

Figure 6. The influence of EPHB2 down-regulation on the growth and migration of gastric cancer cells. A. EPHB2 expression was suppressed by transfection of EPHB2 siRNA (siEPHB2) pool into MKN-28 and MKN-45. Immunoblot analysis was performed using the antibodies indicated in the figure. B. MKN-28 and MKN-45 were transfected with siEPHB2 or control, and growth assay was performed using the Cell counting Kit-8. C. The effect of EPHB2 down-regulation on the migration of MKN-28 and MKN-45 was evaluated by wound healing assay. Yellow dashed lines indicate the approximate edge of the wound. Data are presented as the mean ± SD. Ctl, control, $^{****}P<0.0001$.

EPHB2 belongs to a group of ISC signature genes that are highly expressed in the basal cells of intestinal crypts [25]. Thus, it is not surprising to observe the specific expression of EPHB2 at the bases of intestinal metaplasia, which has already been shown in our previous study [26]. Ectopic CDX2 expression plays a key role in the intestinal phenotype of GCs [27]. We investigated the correlation between SC-related markers and CDX2 expression and noted that all ISC markers had a strong positive correlation with CDX2, while none of the CSC markers were significantly correlated with CDX2 expression. Furthermore, we discovered that both EPHB2 mRNA and protein levels were directly enhanced by CDX2 induction in GC cell lines. These findings suggest that the close relationship between EPHB2 and the intestinal phenotype that develops from intestinal metaplasia persists during GC development.

We also found that EPHB2 expression in GCs was positively correlated with other ISC genes, such as *OLFM4*, *LGR5*, and *EPHB3*, implying intimate co-expression of ISC signature genes in the normal stem cell niche is maintained during GC development. This correlated expression pattern of ISC genes has also been observed in CRCs [18, 25]. Some ISC genes in the normal intestinal epithelium have been suggested to have potential as CSC markers in CRC, since most cancers originate from normal stem/progenitor cells. Indeed, Suarez et al. demonstrated that EPHB2 sorted cells within colorectal tumors display robust tumor-initiating capacity in immunodeficient mice as well as long-term self-renewal potential, suggesting that the ISC program defines a cancer stem cell niche [25]. Thus, it is possible to speculate that EPHB2-positive cells in GCs may have stem cell characteristics, and it would be worthwhile to isolate EPHB2-positive GC cells and explore their potential as cancer stem cells.

EPHB2 expression in GCs was higher than that in matched non-cancerous gastric mucosa, which is consistent with previous studies [28]. This appears to contradict the tumor-suppres-

sive role of in CRCs. However, EPHB2 is not normally expressed in the gastric mucosa, and it appears as intestinal metaplasia develops and substantially increases in gastric adenomas [29]. Therefore, it seems that EPHB2 expression is upregulated in the early stage of cancer development, and declines as cancer cells progress further. This pattern has also been described in colorectal tumors, as mentioned earlier. Yu et al. reported that a reduction in EPHB2 expression was significantly correlated with increased nodal metastasis in patients with GC [15]. In this study, we also found that EPHB2-negative GCs had higher rates of lymph node metastasis and poor clinical outcomes. Notably, multivariate analysis demonstrated EPHB2 as an independent prognostic marker in intestinal-type GCs, but not in diffuse-type GCs. Although CDX2 was significantly associated with better overall survival in both intestinal ($P<0.001$) and diffuse-type GCs ($P = 0.047$) (Supplementary Figure 4), it was not an independent prognostic factor in multivariate analysis (**Table 2**). It is noteworthy that EPHB2 expression, which is partly regulated by CDX2, has a stronger prognostic significance than CDX2. It can be hypothesized that the ISC-related features of EPHB2 may confer a significant prognostic value in intestinal-type GCs.

Based on its prognostic impact, we investigated whether EPHB2 has any functional role in GC progression. We induced EPHB2 overexpression in two GC cell lines, MKN-28 and MKN-74. When examining proliferation and migration abilities, EPHB2 expression significantly attenuated the migration of GC cells, while there was no difference in growth rates. Moreover, EPHB2 downregulation in MKN-28 cells resulted in increased proliferation and migration activities. This result is in line with a previous study by Cortina et al., which showed that EphB-mediated compartmentalization restricts the spreading of tumor cells and suppresses CRC progression [10]. In contrast, Yin et al. demonstrated that EPHB2 promotes migration and invasion in AGS and HGC 27 cell lines [16]. These conflicting findings may be due to variations in cancer types and experimental procedures such as difference in GC cell lines used for functional studies. It is also possible that simple restoration of EPHB2 expression is not sufficient to reverse the biological behavior of GC cell lines that have already progressed to an advanced stage.

To further explain the suppressive effect of EPHB2 on migration, we determined the phosphorylation level of FAK, which has been shown to promote invasive cell phenotype through changes in focal adhesion and cytoskeletal dynamics in various tumors [30]. In addition, FAK phosphorylation was reported to be strongly predictive of gastric cancer recurrence [31]. Indeed, in this study we observed that phosphorylation of FAK significantly changed in GC cells upon EPHB2 overexpression or suppression, indicating that EPHB2-induced alterations in migration activity are likely mediated through FAK signaling. In future studies, it would be interesting to explore the underlying molecular mechanism by which EPHB2 regulates FAK activity in GCs.

Thus, EPHB2 is upregulated in precancerous lesions, intestinal metaplasia and GCs, and is strongly correlated with ISC markers and CDX2 expression. EPHB2 is associated with improved overall survival in GC patients by suppressing migration activity, and multivariate analyses demonstrated that EPHB2 is an independent prognostic factor in intestinal-type GCs.

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Disclosure of conflict of interest

None.

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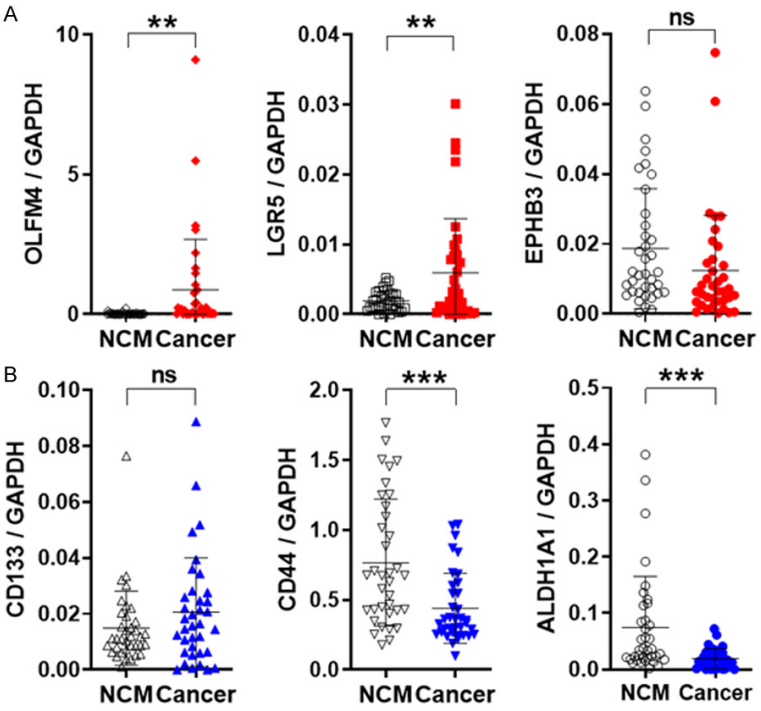
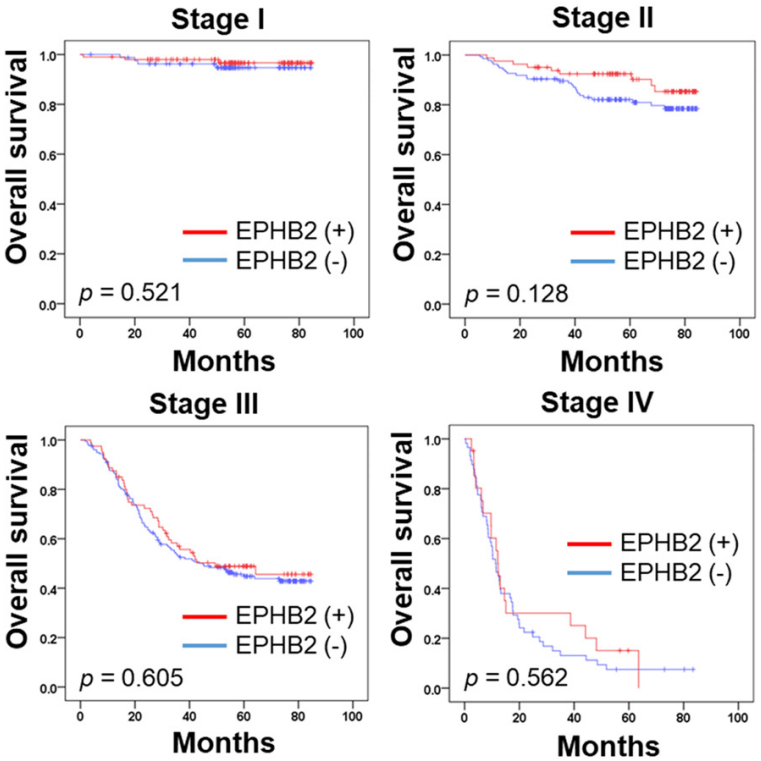


Supplementary Figure 1. mRNA expression of intestinal stem cell markers (*OLFM4, LGR5,* and *EPHB3*) (A) and candidate cancer stem cell markers (*CD133, CD44*, and *ALDH1A1*) (B) from 37 pairs of fresh-frozen gastric cancers and matched non-cancerous mucosa (NCM). Data are presented as the mean ± SD. ns, not significant. $^{**}P<0.01$, $^{***}P<0.001$.

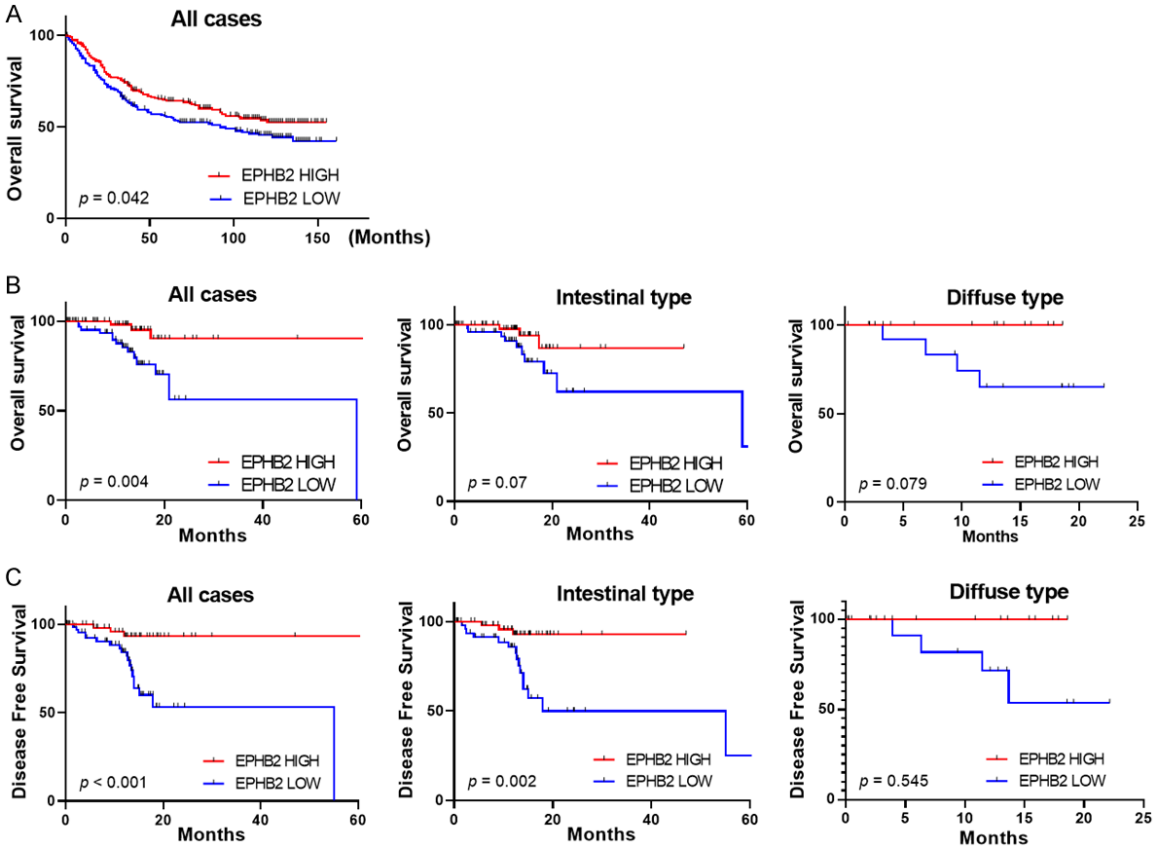


Supplementary Figure 2. Overall survival of gastric cancer patients with EPHB2 expression according to tumor-node-metastasis stages. Stage I (n = 180), stage II (n = 216), stage III (n = 258), and stage IV (n = 79).

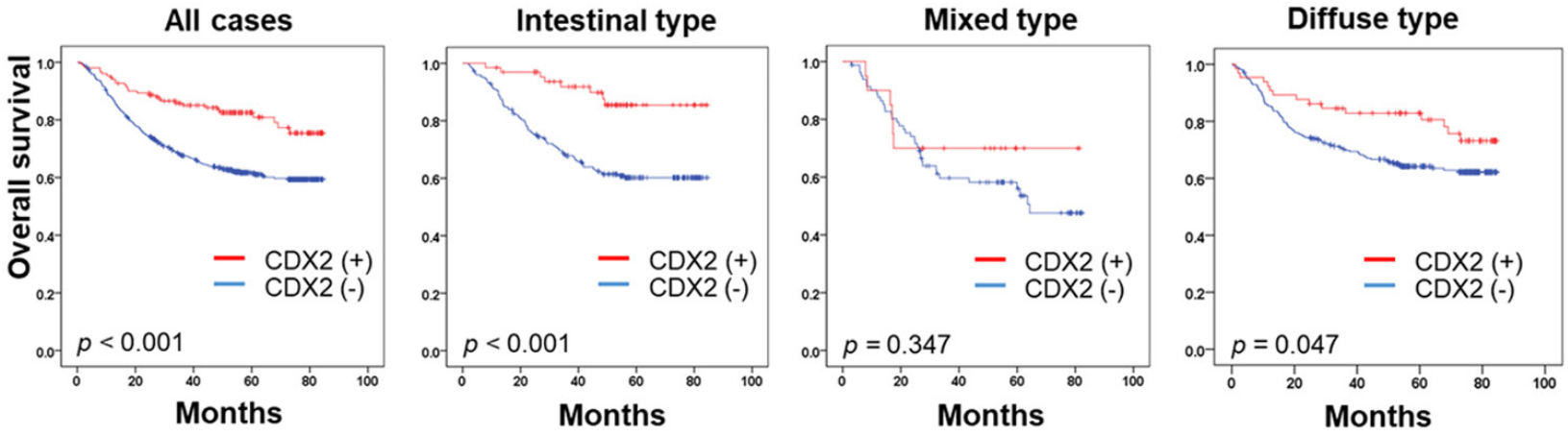
Supplementary Table 1. The results of multivariate analysis for survival rate for all cases

Variables	Category	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value[a]
Age	>65/<65	1.212 (0.934-1.573)	0.148		
Gender	Female/Male	0.973 (0.743-1.274)	0.842		
Location	Upper/Middle/Low/Whole	0.973 (0.814-1.164)	0.764		
Histology	WD/MD/PD/SRC	1.137 (1.031-1.254)	0.010	1.140 (1.021-1.273)	0.020
Lauren classification	Intestinal/Diffuse/Mixed/Unclassified	1.158 (0.972-1.380)	0.101		
Lymphatic invasion	Positive/Negative	3.976 (2.791-5.664)	<0.001	1.580 (1.075-2.323)	0.020
Venous invasion	Positive/Negative	3.690 (2.828-4.816)	<0.001	1.478 (1.103-1.980)	0.009
Perineural invasion	Positive/Negative	3.044 (2.267-4.088)	<0.001	1.199 (0.879-1.635)	0.253
Tumor stage	IV/III/II/I	3.941 (3.319-4.680)	<0.001	3.202 (2.617-3.916)	<0.001
CDX2	Positive/Negative	0.442 (0.294-0.664)	<0.001	0.738 (0.483-1.127)	0.159
EPHB2	Positive/Negative	0.580 (0.439-0.766)	<0.001	0.862 (0.645-1.153)	0.316

HR, Hazard ratio; CI, confidence interval. [a]Cox proportional hazard model.



Supplementary Figure 3. Validation of prognostic significance of EPHB2 in TCGA and GSE84437 cohorts. (A) Overall survival of EPHB2 low (*n* = 217) and high (*n* = 217) gastric cancers (GCs) with GSE84437 database. Overall (B) and disease-free survival (C) analyses for EPHB2 in GC patients with TCGA database. All cases (*n* = 67), intestinal type (*n* = 53), and diffuse type (*n* = 14).



Supplementary Figure 4. Survival analysis for CDX2 in gastric cancer patients. All cases (*n* = 743), intestinal (*n* = 291), mixed (*n* = 102), and diffuse type (*n* = 346) gastric cancers.