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Expression of intestinal stem cell and cancer stem cell markers in submucosal invasion and its prognostic significance in gastric cancers

(Running head: stem cell markers in gastric cancer)

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Abstract

Submucosal invasion is a critical step in gastric cancer (GC) progression, which greatly enhances metastasis risk. Cancer stem cells are responsible for invasion, metastasis, and tumor growth. To identify stem cell-related markers associated with submucosal invasion in GCs, we investigated the expression of candidate cancer stem cell (CSC) markers (CD133, CD44, and ALDH1A) and intestinal stem cell (ISC) markers (EPHB2, OLFM4, and LGR5) in early GCs with submucosal invasion. Remarkably, expression of all ISC markers and CD133 was frequently confined to the basal area of the lamina propria (basal pattern) in mucosal cancer. The proportion of stem cell marker-positive cells substantially increased during submucosal invasion. Given that ISC markers are restricted to the crypt base of the normal intestinal mucosa, these findings suggest that many early GCs may retain hierarchical characteristics. CD44 expression showed a focal pattern, ALDH1A was predominantly expressed diffusely, and there was no expansion of CD44 or ALDH1A expression in the submucosal cancer cells. RSPO2 from muscularis mucosa seem to be partly responsible for the increased expression of ISC markers in GC cells at the basal areas. We also found that ISC markers were correlated with CDX2 expression in GCs, indicating that ISC markers are involved in the intestinal differentiation in GCs. Interestingly, ISC markers (EPHB2 and OLFM4) and CD133 showed a positive impact on clinical outcomes. In particular, the prognostic value of EPHB2 was significant for intestinal-type GCs in a multivariate analysis. In summary, ISC markers and CD133 showed a basal distribution pattern along with enhanced expression in submucosal invading cells in early GCs. EPHB2 was an independent prognostic marker in intestinal-type GCs.

Keywords: Gastric cancer, Submucosal invasion, Intestinal stem cell, Cancer stem cell, Prognosis.

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1. Introduction

The cancer stem cell (CSC) model suggests that tumor growth is induced by a small group of self-sustaining cells with longevity, infinite proliferation, and the ability to differentiate into a heterogeneous population of tumors [1]. The subpopulation at the top of the hierarchy, responsible for tumor formation, maintenance, and sustained growth, is essential to the CSC model [2]. The expression of distinct combinations of cell surface markers is an important tool in the identification and isolation of CSCs. Several candidate CSC markers have been identified for gastric cancer (GC), including CD133, CD44, and aldehyde dehydrogenase 1 (ALDH1) [3]. CD133 is a transmembrane glycoprotein that is believed to be a CSC marker in various solid tumors. Although research has demonstrated CSC properties in the CD133 subpopulation, there is controversy regarding the utility of CD133 as a CSC marker [4-6]. CD44 is also a transmembrane glycoprotein with various biological roles, and the first evidence of gastric CSCs was the self-renewal and heterogeneous lineage of a CD44+ subpopulation [7]. CSC populations in GC have been identified using a combination of CD44+ and other markers, including EPCAM [8,9], CD54+[10], and CD24 [11]. Additionally, ALDH1 functions as a regulator of cell proliferation and stem cell differentiation and a marker of CSCs in a variety of cancers [3]. ALDH1+ cells isolated from gastric cancers have CSC properties [12,13].

The epithelial lining of the small intestine represents a prototype example of a mammalian stem cell-driven self-renewal tissue. A lineage-tracing experiment identified a leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) as a potent intestinal stem cell (ISC) marker [14], and ISCs were subsequently identified as the cellular origin of intestinal tumors [15]. Combinatorial microarray and proteomic approaches have discovered additional markers of LGR5+ stem cells, such as the ephrin type-B receptor 2 (EPHB2), Olfactomedin 4 (OLFM4), SPARC-related modular calcium-binding 2 (SMOC2),

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and ring finger protein 43 (RNF43) [16,17]. In addition, LGR5+ cells act as CSCs to induce tumor growth in human colorectal cancers [18,19]. Most gastric adenomas and adenocarcinomas occur in the background of intestinal metaplasia (IM). We have previously demonstrated the appearance of cells expressing ISC markers, LGR5, ASCL2, EPHB2, and OLFM4 in IM and gastric adenoma samples, suggesting these markers have a functional relevance in early gastric carcinogenesis [20,21]. Indeed, LGR5+ cells in the gastric antrum are of cellular origin in gastric adenomas and intestinal-type GCs in mice [22]. It is reasonable to consider these ISC markers as candidate CSC markers in human GCs.

An increasing number of studies suggest that CSCs are also responsible for tumor migration and invasion. Metastatic cancer cells of most carcinomas tend to recapitulate the organization of their primary tumors, indicating the cells with stem cell properties in the migrating or invading cancer cells [23]. In GC, crossing the muscularis mucosa (MM), a thin muscular layer that separates the mucosa from the submucosa, is a critical invasive step. This increases the risk of metastases, as the submucosal space contains substantial lymphatic and venous vessels. Submucosal invasion in GC is frequently detected by identifying small cancer cell clusters with intact MM, allowing for the comparison of SC marker expression between mucosal and submucosal cancer cells. However, no one has investigated the differential SC marker expression during submucosal invasion in GC. In this study, we examined the expression of ISC and CSC markers in GC cells infiltrating the submucosal space and investigated the prognostic significance of these SC markers in a large cohort of GC patients.

2. Materials and methods

2. 1. Participants

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Formalin-fixed and paraffin-embedded (FFPE) early GCs were collected from 91 patients who underwent surgical resection or endoscopic submucosal dissection at Jeju National University Hospital, Jeju, Korea, from 2008 to 2016. Sixty seven cases (74%) of them had submucosal invasion and lymph node metastasis was observed in 20 cases (20%). The clinicopathological features are shown in Supplementary Table S1 and their associations with lymph node metastasis are in Supplementary Table S2. For survival analysis, 840 FFPE GCs were obtained from the patients who underwent curative gastrectomy at Seoul National University Hospital, Seoul, Korea, from 2004 to 2005 [24]. Clinicopathological data such as patient age, gender, histological type, evidence of lymphatic invasion, and TNM pathological stages were obtained by thoroughly reviewing the medical and pathological records. In addition, 37 paired, fresh-frozen GC tissues and matched non-cancerous gastric tissues were provided by the Jeju National University Hospital Biobank, a member of the National Biobank of Korea, for which informed consent was obtained from all subjects. This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. H-1209-037-424) and Jeju National University Hospital (IRB No. 2016-10-001) respectively, and all procedures were in accordance with the Helsingki Declaration of 1964 and later versions.

2.2. Tissue microarray construction

In total, four tissue microarrays (TMAs) containing 91 early GCs form Jeju National University hospital and 14 TMAs containing 840 GCs from Seoul National University Hospital were generated. 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 core (4 mm in diameter for 91 early GCs, 2 mm for 840 GCs) was extracted from individual FFPE gastric tumors (donor blocks) and placed in a new recipient paraffin block (tissue array block) using a trephine apparatus (SuperBioChips

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Laboratories, Seoul, Korea).

2.3. Immunohistochemistry interpretation

Immunohistochemistry was carried out 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 [25]. The primary antibodies used were anti-EPHB2 (R&D systems, Minneapolis, MN, USA; 1:700), anti-OLFM4 (Abcam, Cambridge, UK; 1:500), CD133 (Abnova, Taipei city, Taiwan; 1:50), CD44 (Novocastra Laboratories Ltd., Newcastle, UK, 1: 100), ALDH1A (BC Biosciences, USA, 44/ALDH; 1:800), CDX2 (BioGenex, CA, USA, 1:300) and anti-β-catenin (Novocastra Laboratories Ltd., Newcastle, UK; 1:800). For 91 early GCs, distribution of SC marker expression was assessed and classified into three categories; basal, focal, and diffuse patterns. Basal pattern denotes SC marker expression confined to the lower one third of cancers in the mucosa. Focal pattern denotes that more than 5% of cancer cells positive in a patched pattern, and diffuse pattern denotes that more than 30% of cancer cells are positive in a diffuse manner. The intensity of SC marker expression was divided into three scores; 1 (weak), 2 (moderate), and 3 (strong). EPHB2, CD133, and CD44 were considered as positive when more than 10% of tumor cells show membranous staining with moderate or strong intensity. For OLFM4 and ALDH1A, it was considered as positive when more than 10% of tumor cells show cytoplasmic positivity with moderate or strong intensity. The positivity criteria for each marker is based on the previous studies [26-28]. Representative images of IHC for each SC marker are shown in Supplementary Figure S1. For β-catenin and CDX2, it was considered as positive when more than 10% of the tumor cell nuclei were strongly stained.

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2.4. RNA in situ hybridization and interpretation

In situ hybridization (ISH) for LGR5, RSPO2, and RSPO3 was performed using RNAscope FFPE assay kit (Advanced Cell Diagnostics, Inc., Hayward, CA, USA) as described previously [20]. Briefly, 4-μm tissue sections of TMA are pretreated with heat and protease digestion followed by hybridization with the LGR5, RSPO2, and RSPO3 probe. Then, an HRP-based signal amplification system is hybridized to the probe before color development with 3,3′-diaminobenzeidine tetrahydrochloride (DAB). The housekeeping gene ubiquitin C (UBC) and the bacterial gene DapB served as positive and negative controls, respectively. Positive staining was identified as brown punctate dots in the nucleus and/or cytoplasm. Expression of LGR5 was quantified according to the manufacturer's scoring guideline: score 0, no staining or less than one dot per cell; score 1: 1 to 3 dots per cell (visible at ×20–40 magnification); score 2: 4 to 10 dots per cell and no or very few dot clusters (visible at ×20–40); score 3: > 10 dots per cell and fewer than10% positive cells have dot clusters (visible at ×20); score 4: > 10 dots per cell and >10% of positive cells have dot clusters (visible at ×20) [25].

2.5. 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 the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 - 2μg) was subjected to reverse transcription with the GoScript reverse transcription system (Promega, Madison, Wisconsin, USA). Quantitative RT-PCR reactions were performed with Premix EX Taq (Takara bio, Shiga, Japan) according to the manufacturer's instructions. Cycling conditions were followed: 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) [25]. The data were analyzed using the 7500 system SDS (Ver.1.4) software (Applied Biosystems). The

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TaqMan gene expression assays were used 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 the endogenous control.

2.6. Gastric cancer cell lines and in-vitro assay

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 % CO2. Recombinant human Wnt3a (Cat # 5036-WN-010) and GREM1 (Cat. # 5190-GR-50) proteins were purchased from R&D, and RSPO2 (Cat. # 120-43) and RSPO3 (Cat. # 120-44) proteins were purchased from Peprotech (Rocky Hill, NJ, USA). For in vitro assay, MKN-45 cells were harvested and seeded at 5 x 10^3 cells /well on 12-well plates and incubated in a serum-free media at 37°C. After 24 hours, GREM1 (2μg/ml), RSPO2 (1μg/ml), and RSPO3 (1μg/ml) together with Wnt3a (1μg/ml) were added to the media. Three days after treatment, cells were harvested and subjected to RT-PCR. Experiments were carried out 3 times independently.

2.7. Transfection of CDX2

Transfection was performed previously described [25]. Cells were plated at 1 × 10^6 cells per well (6-well plate) one day before transfection. Full-length cDNA encoding CDX2 cDNA (pCMV6-CDX2) was purchased from OriGene (Rockville, MD, USA). The cells were transfected with 2.5 μg of pCMV6-CDX2 using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA) according to the protocol

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of manufacturer. pCMV empty vector was used as a control vector. One day after transfection, cells were subjected to real-time PCR.

2.8. Western blot analysis

Cellular proteins were isolated using lysis buffer (iNtRON Biotechnology, Seongnam, Korea) and were quantitated with BCA protein assay kits (Pierce, Rockford, IL, USA). Cell lysates were run on a 10% SDS-polyacrylamide gel and were transferred to a PVDF 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 hour and then incubated with specific primary antibodies; CDX2 (BioGenex), EPHB2 (R&D systems), and HSP90 (Origene, Rockville, MD, USA) for overnight at 4 °C After washing with TBS containing 0.1% Tween-20, the membrane was incubated for 1 hour with secondary antibodies. Alliance-Mini.HD9 chemiluminescence documentation system (UVItec Cambridge, UK) was used to visualize the target proteins.

2.9. 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). The correlation between SC marker expression and lymph node metastasis were tested using Pearson's chi-square test. Between-group comparisons were performed using Student's t-test or Turkey's multiple comparison test. The correlations between SC markers expression and CDX2 were evaluated by 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 for comparing hazard ratios in multivariate analyses. A

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P-value < 0.05 was considered statistically significant.

3. Results

3.1. Distribution of intestinal stem cell (ISC) and cancer stem cell (CSC) markers in early gastric cancers

We hypothesized that cancer cells with stem cell features might be responsible for submucosal invasion in early gastric cancer progression. If so, cancer cells in the submucosal space are enriched with cells expressing CSC markers. We performed immunohistochemistry or RNA ISH for ISC markers (EPHB2, LGR5, and OLFM4) and candidate CSC markers (CD133, CD44, and ALDH1A) on tissue microarrays containing 91 early GCs to investigate the expression profile of SC-related markers in submucosal invasion. First, we assessed the distribution patterns of all markers in the mucosa and discovered that the most frequently observed pattern for all ISC markers in early GCs was expression restricted to the mucosal base (EPHB2: 57%, LGR5: 58%, OLFM4: 30%,), reminiscent of the normal stem cell architecture (Figure 1a-c). There was no significant difference in the expression intensity, except that OLFM4 expression was weaker in GCs with a focal pattern than in GCs with basal or diffuse patterns. Among CSC markers, only CD133 (34% of early GCs) showed a basal predominant pattern (Figure 2a). CD44 showed a focal expression pattern (49%) and ALDH1A showed a diffuse pattern (57%) (Figure 2b,c). CD133 and CD44 expression were stronger in GCs with a diffuse pattern than in GCs with a focal pattern. Notably, we found many GCs with a basal distribution pattern of multiple SC markers (Supplementary Figure S2). Among all ISC and CSC markers, only EPHB2 showed a significant association with lymph node metastasis; early GCs with diffuse EPHB2 expression had no lymph node metastasis. This finding suggests that EPHB2 might be a predictive marker for lymph node metastasis

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in early GCs (Table 1).

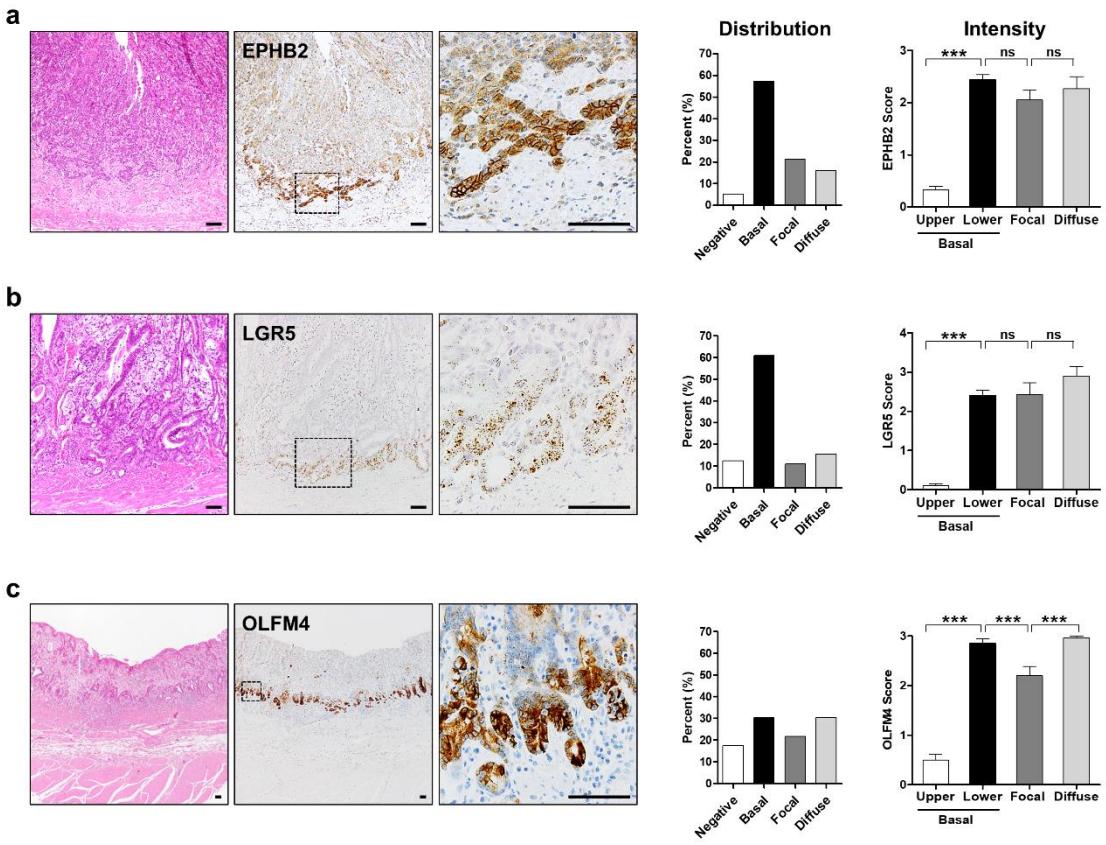

Figure 1. Basal distribution of intestinal stem cell markers in early gastric cancers (GCs). Immunostaining or RNA *in situ* hybridization for EPHB2 (**a**), LGR5 (**b**), and OLFM4 (c) in early GCs (n = 91). The distribution patterns and intensity of each marker are shown in bar graphs. Black boxed areas in the middle are shown at higher magnification in the images on the right. ns, not significant. ***p < 0.001. Scale bars: 50 μm.

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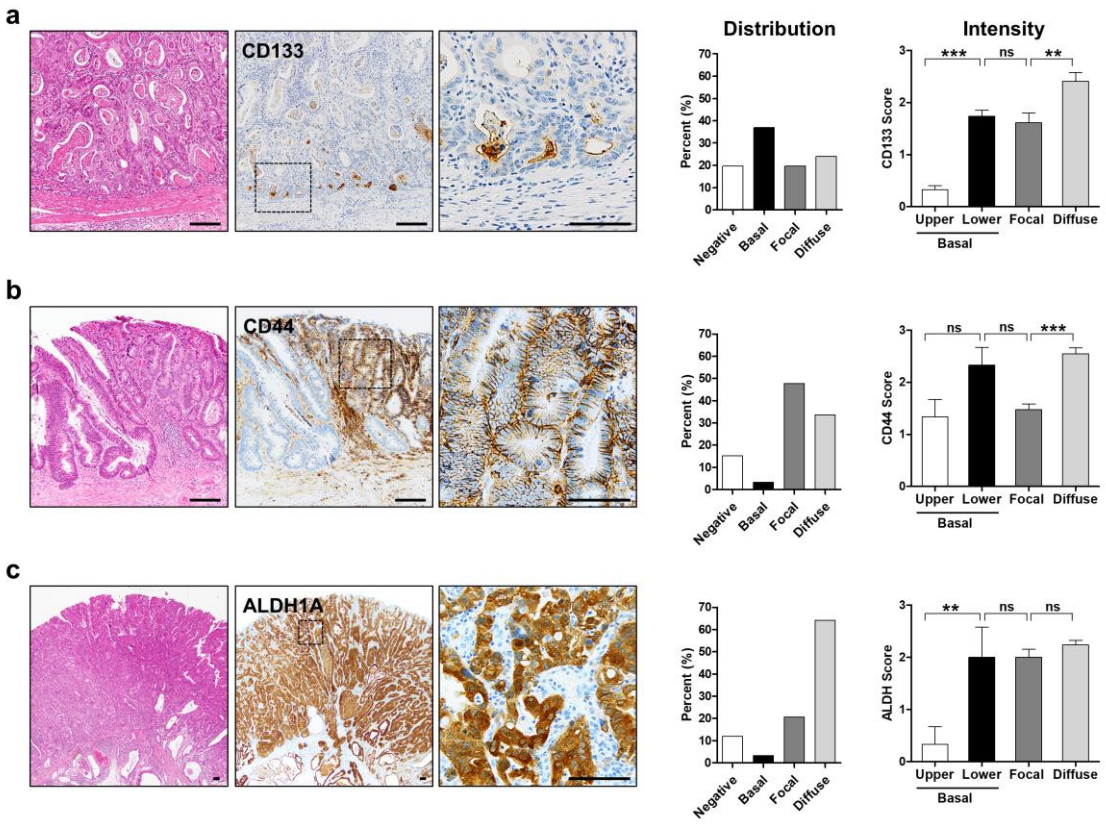

Figure 2. Distribution of cancer stem cell markers in early gastric cancers (GCs). Immunostaining of CD133 (**a**), CD44 (**b**), and ALDH1A (**c**) in early GCs (n = 91). The distribution patterns and intensity of expression are shown in bar graphs. Black boxed areas in the middle are shown at higher magnification in the images on the right. ns, not significant. $^{**}p < 0.01$; $^{***}p < 0.001$. Scale bars: 50 μm.

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Table 1 Association of intestinal stem cell and cancer stem cell markers with lymph node metastasis in early gastric cancers

Characteristics	Total No. (%)	Lymph node metastasis		*P*-value
		Negative (%)	Positive (%)	
	74 (100)	59 (80)	15 (20)	
EPHB2				
Negative	3 (4)	1 (33)	2 (67)	
Basal/focal	60 (78)	47 (78)	13 (22)	0.032†
Diffuse	11 (15)	11 (19)	0 (0)	
OLFM4				
Negative	11 (15)	8 (73)	3 (27)	
Basal/focal	42 (55)	35 (83)	7 (17)	0.659†
Diffuse	21 (30)	16 (76)	5 (24)	
LGR5*				
Negative	7 (15)	5 (71)	2 (29)	
Basal/focal	34 (70)	31 (91)	3 (9)	0.351†
Diffuse	7 (15)	6 (86)	1 (14)	
CD133				
Negative	15 (20)	11 (73)	4 (27)	
Basal/focal	40 (56)	33 (83)	8 (27)	0.764†
Diffuse	18 (24)	15 (80)	3 (17)	
CD44				
Negative	10 (14)	7 (70)	3 (30)	
Basal/focal	38 (51)	31 (82)	7 (18)	0.711†
Diffuse	26 (35)	21 (81)	5 (19)	
ALDH1				
Negative	10 (14)	7 (70)	3 (30)	
Basal/focal	21 (29)	18 (86)	3 (14)	0.588†
Diffuse	42 (57)	34 (79)	9 (21)	

* The number of cases evaluable for LGR5 was 48.

† Pearson Chi-square test.

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3.2. Expression of ISC and CSC markers in submucosal gastric cancers

Next, we examined the proportion of SC marker-positive cells and the intensity of marker expression in submucosal cancer cells in comparison with mucosal cancer cells. In GCs with frequently expressing ISC markers (EPHB2, LGR5, and OLFM4) and CD133, the proportion of SC marker-positive cancer cells in the submucosa was significantly higher compared to that in the lamina propria (Figure 3). For instance, as seen in Figure 3a, EPHB2-positive cancer cells are confined to the bottom of the mucosa, whereas submucosal cancer cells predominantly express EPHB2. Interestingly, a few cases of GCs displayed a symmetrical distribution of multiple ISC markers in which expression intensified around the MM, implying the environment niche's influence on ISC marker-positive cancer cells (Supplementary Figure S3). However, in GCs with focal or diffuse patterns of EPHB2 expression, there was no difference in the proportion of EPHB2-expressing cancer cells between the mucosa and submucosa, and similar findings were observed for OLFM4, LGR5, CD133, and ALDH1A (not CD44) (Supplementary Figure S4).

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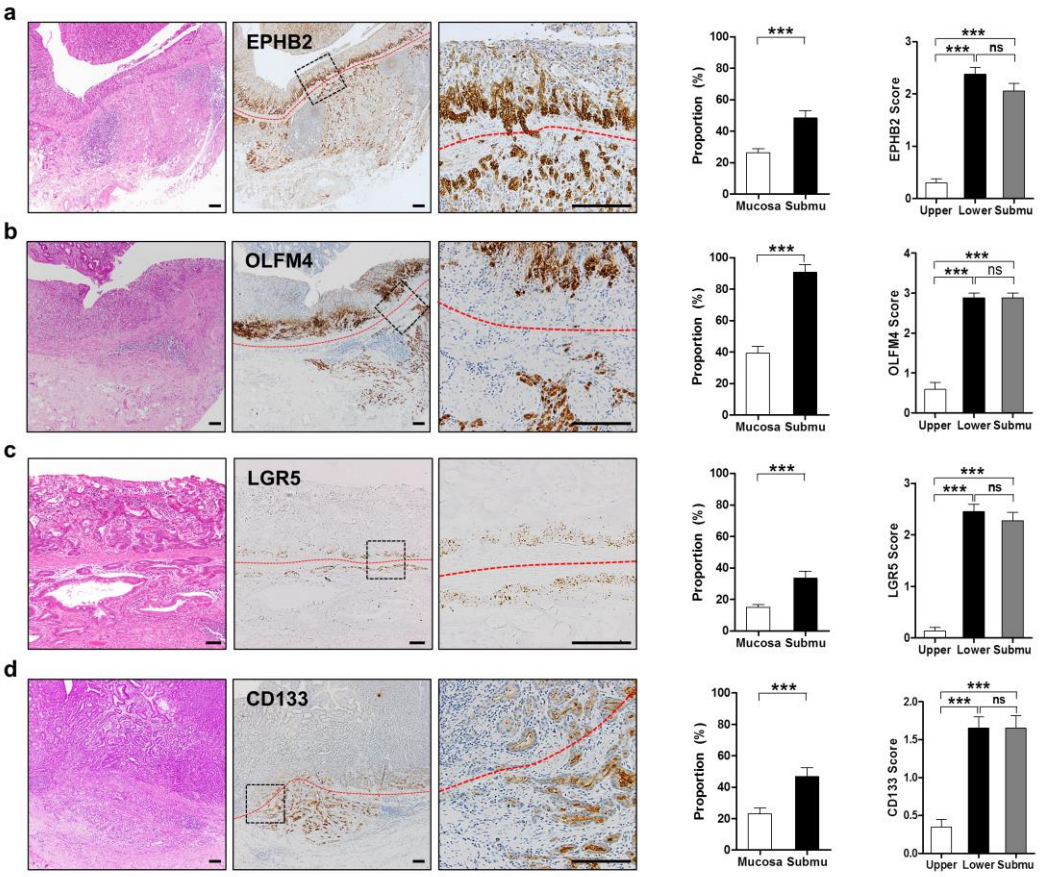

Figure 3. Enrichment of stem cell marker-positive cells in submucosal gastric cancers (GCs) with a basal pattern. Immunostaining of EPHB2 (**a**), OLFM4 (**b**), and CD133 (**d**), and RNA *in situ* hybridization of LGR5 (**c**) in submucosal GCs ($n = 67$). The proportion of stem cell marker-positive cells and intensity of expression in GCs with a basal distribution pattern for each marker are shown in bar graphs. Red dotted lines indicate muscularis mucosa. Black boxed areas in the middle are shown at higher magnification in the images on the right. ns, not significant. ***$P < 0.001$. Scale bars: 100 μm (a, b, and d), 50 μm (c).

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3.3. Wnt3a/RSPO2-induced EPHB2 expression in gastric cancers

Recently, stromal R-spondin3 has been shown to be essential for the stem cell maintenance in the normal gastric mucosa of the mice [29]. In addition, GREM1, an antagonist of BMP signaling, also has been suggested as an important molecule secreted from MM in the colon [30]. Therefore, we hypothesized that these niche factors from MM may lead to upregulation of ISC markers, such as EPHB2 or LGR5, in early GCs. MKN45 was treated with GREM1, RSPO2, and RSPO3 along with Wnt3a to examine which niche factors are involved in the ISC or CSC marker expression. Remarkably, EPHB2 and LGR5 mRNA expression was significantly increased upon RSPO2 and RSPO3 treatment together with Wnt3a (Figure 4a). However, there was no alteration in the expression of OLFM4, CD44, and ALDH1. Next, we performed RNA *in situ* hybridization for RSPO2 and RSPO3 and demonstrated that only RSPO2 is expressed in the smooth muscle cells of MM (Figure 4b). These findings suggest that RSPO2 released from muscularis mucosa of human stomach is likely be responsible for enhanced intestinal stem cell marker expression in early gastric cancer cells.

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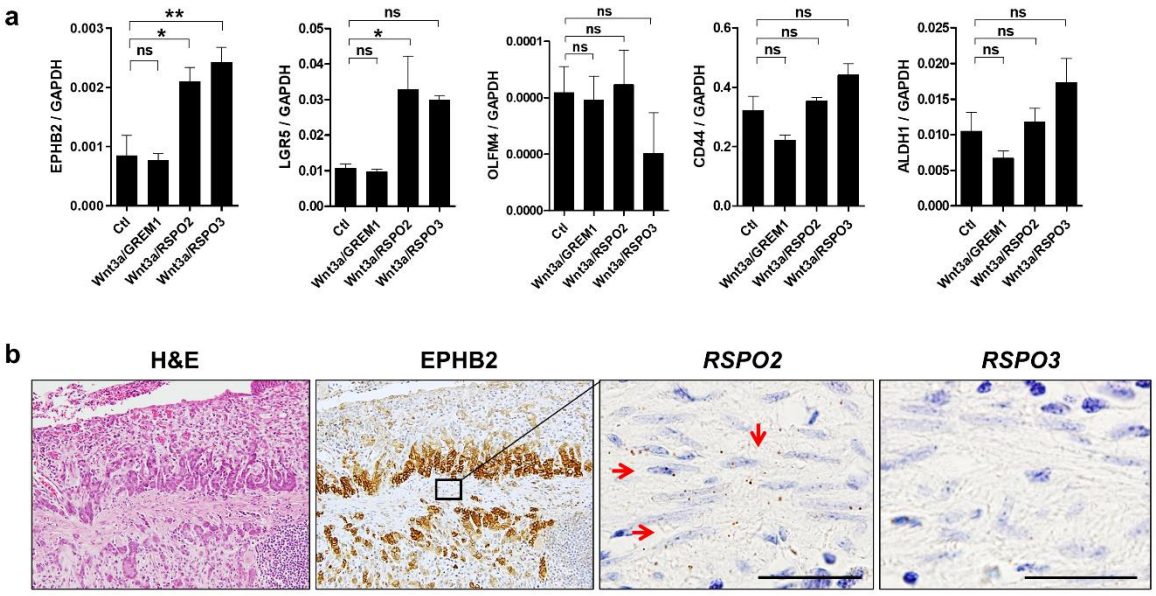

Figure 4. Wnt3a and R-spondin-induced alterations of intestinal stem cell markers in gastric cancer cells. (**a**) Treatment of MKN-45 with niche factors, GREM1 (1μg/ml), RSPO2 (1μg/ml), RSPO3 (1μg/ml) along with Wnt3a (1μg/ml) for 3 days. (b) RNA in situ hybridization for R-spondin 2 and 3 in the muscularis mucosa of gastric cancer with basal EPHB2 expression. Red arrows indicate *RSPO2*-expressing smooth muscle cells. Scale bars: 10μm.

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3.4. Association of ISC and CSC markers with CDX2 in gastric cancers

ISC markers were originally defined in the intestinal and colonic epithelium, and have been recently found in the intestinal metaplasia of the stomach, indicating their close relationship with intestinal phenotype and differentiation. To examine whether these ISC markers are implicated in the intestinal phenotype of GCs, we investigated the association between mRNA expression of CDX2 and ISC markers in GCs. We performed a real-time PCR with 37 pairs of fresh-frozen human GC samples and matched non-cancerous gastric mucosa (NCM), and we noted that ISC marker expression was higher in GCs than in adjacent NCM, whereas CSC marker expression in GCs either showed no significant difference (CD133) or was lower compared to that of the NCM (CD44 and ALDH1A) (Figure 5a). As expected, CDX2 expression was significantly correlated with EPHB2 ($r^2 = 0.31$, $p < 0.001$) and OLFM4 ($r^2 = 0.18$, $p < 0.01$). LGR5 was not significantly correlated ($r^2 = 0.09$, $p = 0.07$) with CDX2 (Figure 5b). However, none of the CSC markers showed positive correlation with CDX2 (CD133: $r^2 = 0.00$, $P = 0.90$; CD44: $r^2 = 0.00$, $P = 0.86$, ALDH1A1: $r^2 = 0.00$, $P = 0.68$) (Figure 5c). EPHB2 expression was positively correlated with OLFM4 and LGR5, but not with CSC markers, confirming an exclusive relationship with ISC markers (Supplementary Figure S5). Additionally, we confirmed the positive correlation between CDX2 and EPHB2 in GC cell lines. An *in vitro* assay demonstrated that CDX2 transfection led to specific upregulation of EPHB2 expression in MKN-28 and MKN-74 GC cells (Supplementary Figure S6), indicating that CDX2 partly regulates EPHB2 expression in GCs.

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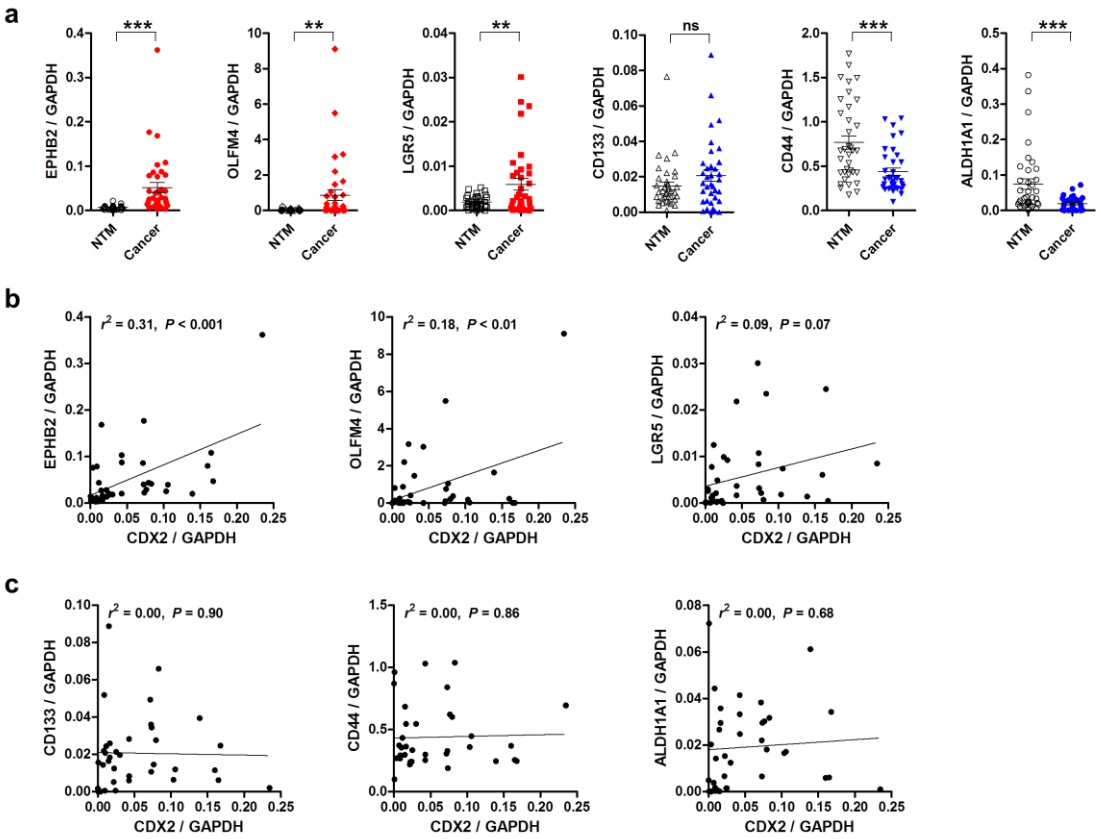

Figure 5. Association of intestinal stem cell (ISC)- and cancer stem cell (CSC) markers with CDX2 in gastric cancers. (**a**) Real time-PCR was performed to measure the expression ISC (EPHB2, OLFM4, and LGR5) and CSC markers (CD133, CD44, and ALDH1A1) with fresh-frozen gastric cancer tissues and their matched non-cancerous mucosa (n = 37). Association of CDX2 with ISC markers (**b**) and CSC markers (**c**) was evaluated.

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3.5. Prognostic significance of intestinal stem cell and cancer stem cell markers in gastric cancer patients

We examined the prognostic value of ISC (EPHB2 and OLFM4) and CSC (CD133, CD44, ALDH1A) markers in a large number of GC patients (n = 706) and found that EPHB2, OLFM4, and CD133 expression was significantly associated with better overall survival (p = 0.000, p = 0.000, p = 0.039, respectively) (Figure 6). However, EPHB2, OLFM4, and CD133were not independent prognostic markers in a multivariate analysis (Supplementary Table S3). Intestinal- and diffuse-type GCs involve different molecular pathways. We therefore separately analyzed the prognostic significance of ISC and CSC markers in intestinal- and diffuse-type GCs. Representative images of EPHB2 expression in the normal glands, intestinal metaplasia, and GCs are shown in Figure 7a-b. Notably, the prognostic value of EPHB2 was significant in intestinal-type GCs (p < 0.001), whereas in diffuse-type GCs, EPHB2 expression was not associated with improved survival (p = 0.176) (Figure 7c). In addition, multivariate analysis revealed that EPHB2 expression is an independent prognostic factor in intestinal-type GCs (HR: 0.520, p = 0.022) (Figure 7d). EPHB2 positivity was significantly higher in GCs with papillary or well-differentiated GCs than in poorly differentiated or signet-ring cell carcinoma (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) than EPHB2-negative GCs. In addition, EPHB2 positivity showed a positive correlation with CDX2 expression

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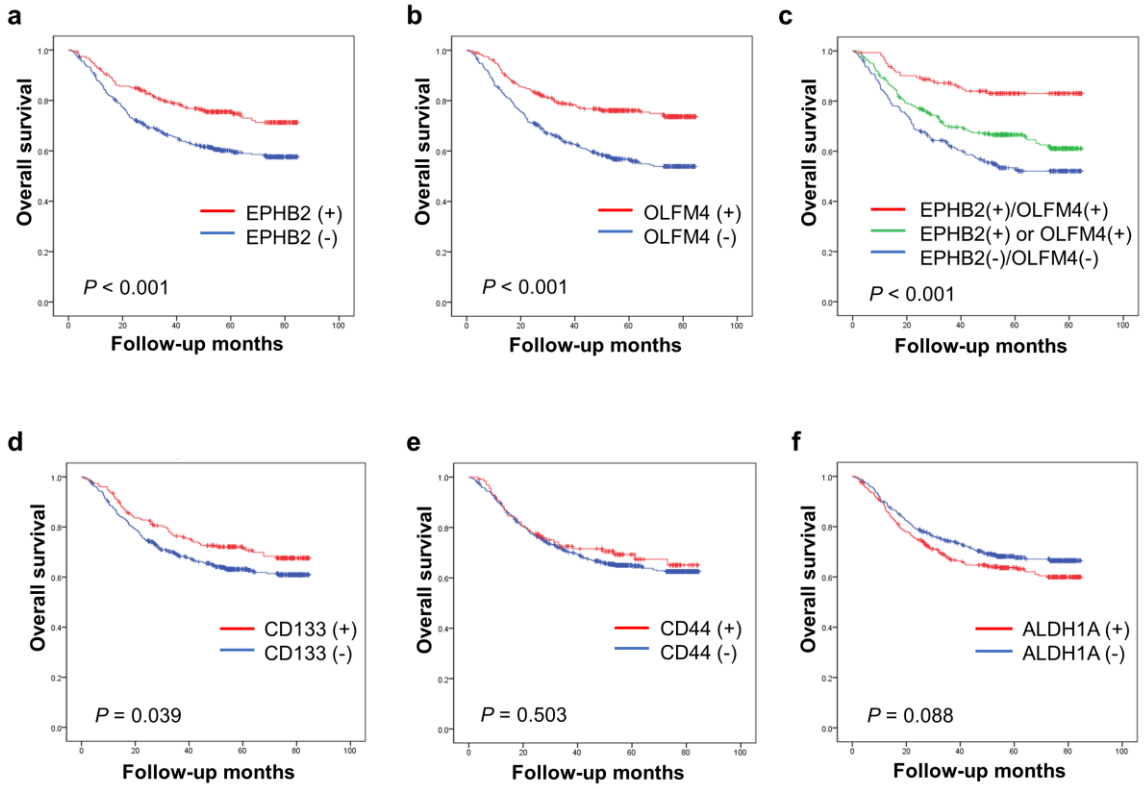

Figure 6. Prognostic significance of intestinal stem cell- and cancer stem cell markers in gastric cancer (GC) patients. Overall survival in GCs (n = 706) according to the expression of EPHB2, OLFM4 (**a**–**c**), CD133 (**d**), CD44 (**e**), and ALDH1A (**f**).

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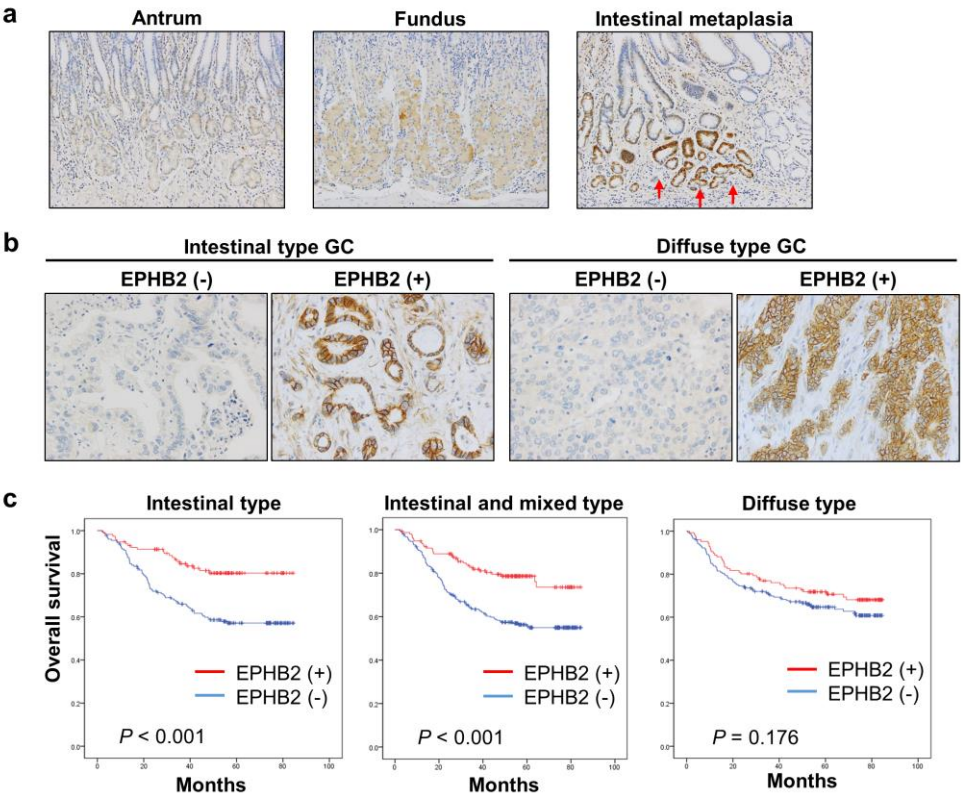

d **Multivariate analysis for survival rate for intestinal-type gastric cancers**

Variables		HR	95% CI	*p*-value[a]
Age	>65/<65	1.107	0.667 - 1.837	0.693
Histology	Poor/Moderate//Well	1.275	0.918 - 1.773	0.148
Lymphatic invasion	Positive/Negative	2.394	1.217 - 4.710	0.011
Venous invasion	Positive/Negative	1.243	0.754 - 2.048	0.393
TNM_7th	IV/III/II/I	4.104	2.844 - 5.924	< 0.001
CDX2	Positive/Negative	0.667	0.268 - 1.658	0.383
CD133	Positive/Negative	1.225	0.693 - 2.164	0.485
OLFM4	Positive/Negative	0.720	0.424 -1.223	0.225
EPHB2	Positive/Negative	0.520	0.298 - 0.909	0.022

Figure 7. Correlations between EPHB2 expression in gastric cancer patients with clinical outcomes according to Lauren classification. (**a**) Representative pictures of EPHB2 expression in the antrum, fundus, intestinal metaplasia. (**b**) Expression of EPHB2 in intestinal and diffuse type gastric cancers. (**c**) Overall survival of gastric cancer patients in intestinal (n = 293), intestinal and mixed (n = 397), and diffuse type (n = 331). (**d**) Multivariate analysis in intestinal type gastric cancers. HR, Hazard ratio; CI, confidence interval. [a] Cox proportional hazard model.

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Table 2 Association between the EPHB2 expression and the clinicopathological characteristics

Characteristics	Total (%)	EPHB2		*p*-value
		Negative (%)	Positive (%)	
Patients	706 (100)	436 (62)	270 (38)	
Age				
≥65	239 (34)	199 (89)	25 (11)	0.086†
<65	467 (66)	299 (64)	168 (36)	
Sex				
Female	225 (29)	143 (64)	82 (36)	0.507†
Male	430 (71)	292 (69)	188 (31)	
Lauren classification				
Intestinal	283 (40)	169 (60)	114 (40)	
Diffuse	318 (45)	199 (63)	119 (37)	0.290#
Mixed	100 (14)	63 (63)	37 (37)	
Undetermined	5 (1)	5 (100)	0 (0)	
Histological				
Well	148 (21)	70 (63)	78 (49)	
Moderate	284 (40)	175 (62)	109 (38)	
Poor	170 (24)	117 (69)	53 (31)	< 0.001#
Signet ring cell	83 (12)	56 (68)	27 (32)	
Others	21 (3)	18 (86)	3 (14)	
Lymphatic invasion				
Negative	250 (35)	137 (55)	113 (45)	0.006†
Positive	456 (65)	299 (66)	157 (34)	
Venous invasion				
Negative	585 (83)	349 (60)	236 (40)	0.013†
Positive	121 (17)	87 (72)	34 (28)	
Perineural invasion				
Negative	312 (44)	167 (54)	145 (46)	0.000†
Positive	394 (56)	269 (68)	125 (32)	
TNM_7th				
I	175 (25)	78 (45)	97 (55)	
II	204 (29)	128 (63)	76 (37)	
III	250 (35)	174 (70)	76 (30)	< 0.001#
IV	77 (11)	56 (73)	21 (27)	
β-catenin				
No nuclear stain	664 (94)	417 (63)	247 (37)	0.032†
Nuclear stain	42 (6)	19 (45)	23 (55)	
CDX2 (n = 704)				
Negative	562 (80)	381 (68)	181 (32)	< 0.001†
Positive	142 (20)	50 (35)	92 (65)	

†Fisher's exact test. #Pearson Chi-square

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4. Discussion

Accumulating evidence suggests that CSCs are responsible for cancer invasion and metastasis. In early GCs, submucosal invasion is a common and critical step for cancer progression that is easily recognized by histologic examination. These features make GCs with submucosal invasion a valuable model to explore CSCs' involvement in the invasion process. However, no study has investigated CSC marker expression in early GCs with submucosal invasion. In this study, we chose three ISC markers and three candidate CSC markers to identify SC markers associated with submucosal invasion in GCs. We found three distinct distribution patterns of SC markers. A basal pattern was predominant for all ISC markers and CD133. A restricted pattern of ISC marker expression is normally found in the intestinal epithelium [31] and at the bottom of tumor structures in colon cancers [32], representing a stem cell hierarchy. Therefore, the observed basal pattern of ISC markers in early GCs suggests that a hierarchical organization fueled by cancer stem cells may be present in the very early stages of cancer progression.

We examined the expression of ISC and CSC markers in submucosal cancer cells and compared them with that of cancer cells in the lamina propria. A schematic diagram of our results is shown in Supplementary Figure S7. Importantly, we noted that all ISC markers and CD133-positive cells are enriched in cells that infiltrate into the submucosa. This expansion was also observed in colon cancer cells, where LGR5 expression was high in invading cells at the tumor-stroma interface [33]. The basal localization of ISC markers and CD133 in early GCs may represent the same distribution pattern as CRC cancer stem cells, considering that the bottom of the mucosa facing the MM is the cancer-stroma interface when gastric cancers cells proliferate and occupy the entirety of the mucosa. However, the submucosal enrichment of ISC marker- positive or CD133-positive cells may be simply due to basal

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localization. We must clarify whether these SC marker-positive cells have any functional advantages over other cancer cells in the submucosal invasion. In CRC, cells expressing high levels of nuclear β-catenin are frequently observed at the invasive fronts and comprised potential migrating CSCs [34]. We therefore sought to examine nuclear β-catenin in the submucosal gastric cancer cells. However, there was no increase in the number of nuclear β-catenin-positive gastric cancer cells at the invasive front.

Retained stem cell-like hierarchy in GCs, as determined by the basal expression of ISC markers, suggests that the differentiation potential is likely to be maintained. As ectopic CDX2 expression plays a key role in the intestinal phenotype of GCs [35], we investigated the correlation between ISC markers and CDX2 expression and noted that all ISC markers had a strong positive correlation with CDX2 expression. However, none of the CSC markers were significantly correlated with CDX2. Additionally, we found that CDX2 overexpression is involved in EPHB2 transcription induction, but not OLFM4 or LGR5 induction. These findings link intestinal differentiation and ISC marker expression in GCs and suggest that ISC marker expression might depend on CDX2 transcription factors. Further research may elucidate the underlying mechanism by which CDX2 regulates the expression of EPHB2, particularly in the cancer stem cell population.

CSC marker expression in cancer tissue is emerging as a clinically-relevant prognostic biomarker in GC management. Its clinical implications are controversial, however, due to variation in experimental procedures and patient populations [3]. In this study, we found that EPHB2, OLFM4, and CD133 are associated with better overall survival, whereas CD44 and ALDH1A expression had no significant impact on clinical outcomes. We previously reported that LGR5 had no prognostic significance in the same cohort of GC patients when we evaluated LGR5 expression by RNA *in situ*

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hybridization and found the positive rate to be low (7%) due to RNA degradation in FFPE samples [36]. It is therefore necessary to perform survival analysis for LGR5 expression when adequate antibodies to human LGR5 for immunohistochemistry becomes available. Interestingly, all markers that have a favorable prognostic significance are SC markers which predominantly exhibited a basal expression pattern in early GCs, and the proportion of marker-positive cancer cells increased in submucosal invasion. Therefore, it seems less likely that SC marker expression has any functional implication in the invasion process or aggressive tumor behavior. Instead, we could hypothesize that although ISC marker-positive or CD133-positive stem cells expand during submucosal invasion, GCs-expressing EPHB2, OLFM4, or CD133 are more likely to sustain hierarchical architecture, representing a relatively more organized or genetically stable state, which in part contributes to improved clinical course.

Crypt bases in the stomach and colon are a stem cell niche environment involving multiple signaling pathways, such as Wnt, Notch, EPH, MYC, and BMP, to regulate stem cell survival and differentiation [30]. Thus, it is possible to speculate that gastric cancer cells at the base of the lamina propria are influenced by several niche factors and acquire stem cell-like phenotypes, leading to ISC marker expression. Several molecules are secreted from stromal cells, myofibroblasts, and smooth muscle cells of MM, including GREM 1, GREM 2, CHRDL1, and RSPO proteins [29,30,37]. We hypothesized that the niche factors released from MM upregulate ISC markers, such as EPHB2 or LGR5, in the basal areas of GCs. To determine which niche factors are involved in ISC or CSC marker expression, MKN-45 cells were treated with GREM1, RSPO2, or RSPO3 along withWnt3a. Remarkably, EPHB2 and LGR5 expression were significantly increased upon RSPO2 and RSPO3 treatment with Wnt3a, while there was no change in OLFM4, CD44, and ALDH1 expression (Supplementary Figure S8a). Interestingly, RNA *in situ* hybridization only confirmed the RSPO2 expression in the MM of

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human stomach (Supplementary Figure S8b), whereas in the mouse stomach and colon only RSPO3 was shown to be expressed in the MM and to play essential roles for stem cell reprogramming [29,37]. Considering the fact that RSPO2 and RSPO3 have common biological activities [38], it is possible that RSPO2 in human stomach plays the same role as RSPO3 in murine stomach.

An EPHB2 reduction accelerates tumorigenesis in the colon of $Apc^{Min/+}$ mice [39], and a loss of EPHB2 expression is a strong indicator of poor overall survival in colorectal cancer (CRC) patients [26,40]. This suggests that EPHB2 has a tumor suppressor role in CRC progression. However, we noted higher EPHB2 expression in GCs compared to matched non-cancerous gastric mucosa, consistent with previous work [41]. Even though this appears to contradict its tumor-suppressive role in CRC, EPHB2 is not normally expressed in the gastric mucosa, but rather it appears as the intestinal metaplasia develops, and substantially increases in gastric adenomas [20]. Both univariate and multivariate analyses have demonstrated that a reduction in EPHB2 expression is significantly correlated with poor overall survival in GC patients [42]. In this study, we also found that a reduction in EPHB2 expression is closely correlated with lymph node metastasis in early GCs and poor clinical outcome in advanced GCs. The prognostic significance of EPHB2 remained in intestinal-type GCs, but not in diffuse-type GCs. This indicates a close relationship between EPHB2 and intestinal differentiation, similar to its correlation with CDX2. Therefore, it appears that EPHB2 expression from precancerous lesions, such as intestinal metaplasia to early gastric cancers, represents a phase during which hierarchical structure and intestinal differentiation are retained and loss of EPHB2 expression occurs as the cancer acquires more oncogenic mutations and progresses to advanced stage.

5. Conclusion

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In summary, the present study identified the distributions of CSC and ISC markers in early-stage GC progression. All ISC markers (EPHB2, OLFM4, and LGR5) and CD133 predominantly demonstrated a basal pattern, confined to the bottom of the mucosa in early GCs, and their expression continued during submucosal invasion. ISC marker expression was strongly correlated with CDX2 and was induced by CDX2 overexpression in GCs. These findings suggest that a significant proportion of GCs with ISC markers retain hierarchical structure with intestinal differentiation potential. These ISC markers and CD133 were associated with improved overall survival in GC patients, and EPHB2 was an independent prognostic marker in intestinal GCs.

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