Prognostic significance of leucine-rich-repeat-containing G-protein-coupled receptor 5, an intestinal stem cell marker, in gastric carcinomas

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Abstract

Background Cells expressing LGR5, an intestinal stem cell marker, have been suggested as cancer stem cells in human colon cancers. Previously, we discovered that LGR5-expressing cells exist in the gastric antrum and remarkably increase in number in intestinal metaplasia. In addition, most gastric adenomas contain abundant LGR5-expressing cells coexpressing intestinal stem cell signatures. However, LGR5 expression in gastric cancers (GCs) and its prognostic significance remain unknown.

Methods We examined the LGR5 expression in GC tissues by real time-PCR and RNA in situ hybridization, and analyzed its clinicopathological relevance and prognostic value. The effects of LGR5 on cancer cell proliferation and migration were assessed with an in vitro transfection technique.

Results LGR5 expression was significantly lower in GCs than in matched nontumorous gastric mucosa. RNA in situ hybridization on tissue microarrays showed that 7 % of GCs were positive for LGR5. LGR5 positivity was associated with old age, well to moderate differentiation, and nuclear β -catenin positivity. Although LGR5 did not show any prognostic significance for all GC cases, it was

associated with poor survival in GCs with nuclear β -catenin expression. LGR5 expression was induced by transfection in GC cell lines with abnormal Wnt activation, which, however, showed no influence on the growth and migration of GC cells.

Conclusion A small portion of GCs expressed LGR5. Although LGR5 was associated with poor survival in GCs with nuclear β -catenin, LGR5 expression in GC cells had no effects on the growth and migration, requiring a further study exploring a biological role of LGR5 in GCs.

Keywords LGR5 · Gastric cancer · In situ hybridization · Prognosis · Stem cells

Introduction

Gastric cancer (GC) is the fifth commonest malignancy in the world and the third leading cause of cancer death worldwide [1]. Despite remarkable progress in the diagnostic and therapeutic tools, patients with advanced GCs still have poor clinical outcomes. The molecular characterization of GCs has led to the identification of several promising targets, including vascular endothelial growth factor receptor 2, c-MET, fibroblast growth factor receptor 1, fibroblast growth factor receptor 2, human epidermal growth factor receptor 2 (HER2), and human epidermal growth factor receptor 3 [2]. Indeed, the anti-HER2 antibody trastuzumab in patients with GC overexpressing HER2 improved overall survival compared with standard chemotherapy [3]. However, the response rates remain in the 25–40 % range across trials, and novel molecularly directed approaches are required [2].

Rapidly mounting evidence suggests that cancer stem cells not only maintain tumor growth but also result in

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relapse after therapy, suggesting they could be a candidate for new targeted therapy [4]. Leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) has been demonstrated as a global stem cell marker in multiple organs, including the small intestine, colon, stomach, hair follicle, kidney, ovary, liver, and mammary gland [5–11]. In these organs, LGR5 not only marks Wnt-driven stem cells that drive constitutive tissue self-renewal, but also defines a class of stem cells induced by tissue damage [11]. LGR5-expressing cells have been shown as the cells of origin of intestinal and gastric adenomas [6, 12]. Moreover, LGR5 has been suggested as a selective marker for human colorectal cancer stem cells [13].

Subsequent studies investigated the clinical and functional implications of LGR5 in a variety of human malignant tumors—for example, colorectal cancers [14–16], GCs [17, 18], and hepatocellular carcinomas [19]. Even in lung adenocarcinomas [20] and gliomas [21], LGR5 expression and its clinicopathological significance were examined although LGR5-expressing stem cells normally do not exist in the lung and brain. In colorectal cancers, the prognostic value of LGR5 was extensively studied and found to be statistically related to the reduced overall survival [22]. However, for GCs, only a limited number of studies have investigated LGR5 expression and its relevance in predicting clinical outcomes [17, 23, 24]. Previously, we specifically identified LGR5-expressing cells in normal gastric mucosa and intestinal metaplastic lesions as well as gastric adenomas by RNA in situ hybridization (ISH) [25]. In this study, we investigated the expression of LGR5 messenger RNA (mRNA) in a large number of GC patients and analyzed its significance in the prognosis of GC patients.

Patients and methods

Patients

Formalin-fixed and paraffin-embedded (FFPE) GCs were collected from 840 patients who underwent curative gastrectomy at Seoul National University Hospital, Seoul, Korea, from 2004 to 2005. We collected clinicopathology data, including patient age and sex, histological type, evidence of lymphovascular invasion, and TNM pathological stages, by reviewing the medical records and pathology records according to the seventh edition of the American Joint Committee on Cancer's cancer staging manual. Also, paired, freshly frozen GC tissues and matched noncancerous gastric tissues were available from 35 GC patients from 2001 to 2005. A normal small intestine specimen was obtained from a patient with gastrointestinal stromal tumor of the small intestine who underwent curative segmental

resection. This study was approved by the Institutional Review Board of Seoul National University Hospital (reference H-1209-037-424).

Gastric carcinoma cell lines

Fifteen human gastric carcinoma cell lines (SNU-1, SNU-5, SNU-16, SNU-216, SNU-484, SNU-601, SNU-620, SNU-638, SNU-668, SNU-719, MKN-1, MKN-28, MKN-45, MKN-74, and AGS) 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. The cell lines were subject to immunocytochemistry for β -catenin after formalin fixation for 1 h.

Tissue microarray construction

Fourteen tissue microarrays containing 840 GCs from gastrectomy specimens were generated. In brief, core tissue biopsies (2 mm in diameter) were obtained from individual FFPE gastric tumors (donor blocks) and arranged in a new recipient paraffin block (tissue array block) with a trephine apparatus (SuperBioChips Laboratories, Seoul, Korea).

Immunohistochemistry

We performed immunohistochemistry on 4- μ m tissue microarray sections using a BOND-MAX automated immunostainer and a Bond Polymer Refine Detection kit (Leica Microsystems, Wetzlar, Germany) according to the manufacturer's instructions. The primary antibody used was anti- β -catenin (Novocastra Laboratories, Newcastle, UK; 17C2; 1:800), and β -catenin staining was considered positive when more than 10 % of the tumor cell nuclei were strongly stained for β -catenin.

RNA ISH and interpretation

ISH for LGR5 was performed with an RNAscope FFPE assay kit (Advanced Cell Diagnostics, Hayward, CA, USA) as described previously [25]. In brief, 4- μ m FFPE tissue sections were pretreated with heat and protease digestion followed by hybridization with LGR5 probe. Then, a horseradish peroxidase based signal amplification system was hybridized to the LGR5 probe before color development with 3,3'-diaminobenzidine tetrahydrochloride. The housekeeping gene ubiquitin C (*UBC*) and the bacterial gene *dapB* served as a positive and a negative control, respectively. Samples with *UBC* easily visible under a $\times 10$ objective lens were considered to be adequate according to the manufacturer's recommendation, and finally 603 GCs

were included for the evaluation. Two gastrointestinal pathologists (B.G.J and G.H.K.) independently interpreted the staining of LGR5. For the discordant cases, a final decision was made by consensus. LGR5 staining was graded on the basis of the percentage of tumor cells positive for LGR5 as follows: grade 0, 0–5 %; grade 1, 5–10 %; grade 2, 10–25 %; and grade 3, 25–100 %. When more than 5 % of tumor cells express LGR5 (more than grade 1), the sample is considered as positive for LGR5.

RNA extraction and quantitative real-time PCR

We prepared total RNA from the 35 paired freshly frozen gastric tumors and corresponding normal gastric tissue samples as well as 15 GC cell lines using TRIzol (Invitrogen, Carlsbad, CA, USA). Total RNA (1 µg) was reverse transcribed with oligo-dT primers and the GoScript reverse transcription system (Promega, Madison, WI, USA). PCRs were performed with Premix EX Taq (Takara bio, Shiga, Japan) according to the manufacturer's recommendations, and the cycling conditions were followed: initial denaturation for 30 s at 95 °C, followed by 40–50 cycles of 95 °C for 5 s and 60 °C for 34 s in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The data were analyzed by the 7500 real-time PCR system software, SDS (version 1.4; Applied Biosystems). The TaqMan gene expression assays used were as follows: Hs00173664_m1 (*LGR5*), Hs00362096_m1 (*EPHB2*), Hs00270888_s1 (*ASCL2*), Hs00197437_m1 (*OLFM4*), and Hs0275899_g1 (*GAPDH*). *GAPDH* served as the endogenous control, and all experiments were performed in duplicate.

Western blot analysis

Cellular proteins were extracted from the GC cells in lysis buffer (iNtRON Biotechnology, Seongnam, Korea), and protein levels were determined by bicinchoninic acid protein assay kits (Pierce, Rockford, IL, USA). The rabbit anti-LGR5 (Abcam, Cambridge, UK; ab75850) and mouse anti- α -actin (Sigma-Aldrich, St Louis, MO, USA) antibodies were used as primary antibodies. After overnight incubation at 4 °C and washing with tris(hydroxymethyl)aminomethane-buffered saline containing 0.1 % Tween 20, blots were incubated for 1 h at room temperature with secondary antibodies, and then washed and visualized with enhanced chemiluminescence kits (Pierce).

Transfection with LGR5

Full-length complementary DNA encoding LGR5 (pEX-LGR5) was purchased from GeneCopoeia (Rockville, MD, USA). Cells were seeded at 1×10^6 cells per well in a six-well plate and transfected with 2.5 µg of pEX-LGR5 or

control vector (pEX-EGFP) by means of Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. One day after transfection, cells were subjected to reverse transcription PCR (RT-PCR), Western blot, and a proliferation assay.

Cell proliferation assay

Twenty-four hours after transfection in a six-well plate, cells were harvested and seeded at 5×10^3 cells per well on 96-well plates and incubated at 37 °C. To examine the effect of LGR5 on the chemosensitivity, 5-fluorouracil (Sigma-Aldrich) was added at a concentration of 100 µg/ml and vehicle (dimethyl sulfoxide) was added to control cells. After addition of 10 µl of Cell Counting Kit-8 reagent (Dojindo, Tokyo, Japan) to each well and incubation for 1 h, the absorbance was measured at 450 nm with a spectrophotometer (Thermo Labsystems, Beverly, MA, USA).

Wound healing assay

AGS and SNU-484 cells were cultured in six-well plates until they were confluent after transfection with the control vector or LGR5. The monolayer was scratched with a pipette tip to create a wound and washed twice with culture medium to remove cell debris. Cellular migration was monitored and photographed at 0 and 48 h.

Statistical analysis

Statistical analyses were performed with the statistical software program PASW 18.0 (IBM SPSS Statistics, Chicago, IL, USA) and Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The correlations between LGR5 positivity and clinicopathological parameters were tested by Fisher's exact test or Pearson's chi-square test. Between-group comparisons of the real-time PCR data were performed by Student's *t* test. Survival curves were estimated by the Kaplan–Meier method, and the log-rank test was used to compare groups. The results were considered significant when $P < 0.05$.

Results

LGR5 expression in human GC

To compare the expression of LGR5 between human GCs and noncancerous tissues, real-time PCR was done on a series of 35 pairs of freshly frozen human GC samples and matched noncancerous gastric mucosa. When we evaluated individual cancers, LGR5 expression

of GC was lower than that of noncancerous tissues in most patients (80 %, 28 of 35 cases) (Fig. 1a). Overall, GCs expressed a significantly lower level of *LGR5* mRNA than adjacent noncancerous gastric tissues ($P < 0.01$) (Fig. 1b). We previously demonstrated that *LGR5*-expressing cells in gastric adenomas coexpress other intestinal stem cell (ISC) signatures such as *ASCL2*, *EPHB2* and *OLFM4*, which we thought suggested a stem cell feature of *LGR5*-expressing cells. To see whether GCs retain this relationship of *LGR5* with ISC markers, we compared the expression levels of the ISC markers between *LGR5*-low and *LGR5*-high groups, which were divided by the value of 0.1 (relative mRNA level of *LGR5* to *GAPDH*). Only *EPHB2* expression was slightly higher in the *LGR5*-high group than in the *LGR5*-low group (Fig. 1d). No difference was observed in the expression of *OLFM4* and *ASCL2* between groups (Fig. 1c, e), indicating a reduced connection of *LGR5* with ISC markers in GCs.

Association of *LGR5* expression with clinicopathological parameters in GC

RNA ISH on a small intestine clearly demonstrates specific marking of *LGR5*-expressing stem cells at the crypt bases as seen previously (Fig. 2a–c) [5]. No nonspecific staining was observed in any stromal cells or intestinal epithelial cells above crypt bottoms. RNA ISH was performed on the tissue microarrays containing 840 GCs. Finally, 603 cases were included for the analysis. In total, 42 cases (7 %) of GCs were positive for *LGR5* (Fig. 2d–f). The clinicopathological relevance of *LGR5* expression is shown in Table 1. *LGR5* positivity was significantly higher in older age (older than 65 years) ($P = 0.002$), and GCs with well-differentiated or papillary histological type expressed *LGR5* more frequently than did poorly differentiated or signet ring cell carcinoma ($P = 0.026$). Immunohistochemical analysis for β -catenin was also performed to investigate the relationship between *LGR5* and Wnt

Fig. 1 *LGR5* expression in gastric cancers and its correlation with other intestinal stem cell markers. **a** Real-time PCR analysis examined the expression of the intestinal stem cell markers *LGR5*, *ASCL2*, *EPHB2*, and *OLFM4* with 35 pairs of gastric cancers and

corresponding noncancerous gastric mucosa. **b–e** The mean level of *LGR5* in gastric cancers was significantly lower than in noncancerous gastric tissues, and was associated with *EPHB2* expression, but not with *OLFM4* and *ASCL2* expression. *n.s.* not significant

Fig. 2 RNA in situ hybridization (ISH) for LGR5 in gastric carcinomas. **a–c** RNA ISH on a specimen of human small intestine specifically identified LGR5 cells (*arrows*) at the bases of crypts. RNA ISH on tissue microarrays containing 840 gastric cancers was

performed, and tumors were interpreted according to the percentage of LGR5 positive cells. **d** Grade 1, **e** grade 2, **f** grade 3. **a, b** $\times 100$; **c–f** $\times 400$

pathway activation. LGR5 positivity showed a strong positive correlation with nuclear β -catenin expression (Fig. 3). However, LGR5 revealed no correlation with sex, location, lymphatic invasion, venous invasion, and TNM stage.

Prognostic value of LGR5 in GC

Next, we assessed the prognostic significance of LGR5 in GC patients using Kaplan–Meier analysis and found that LGR5 positivity had no correlation with overall survival ($P = 0.560$) (Fig. 4a). Recently, it has been shown that LGR5 functions as a ligand for R-spondins and potentiates the Wnt/ β -catenin pathway only when Wnt signaling is activated [26, 27], which led us to speculate that LGR5 expression could exert a synergistic effect on GCs harboring an abnormal Wnt pathway. Therefore, we tried to examine the prognostic effect of LGR5 in a subgroup of GC patients who had nuclear β -catenin expression ($n = 34$), indicative of enhanced Wnt signaling. Nuclear β -catenin positivity itself was not a prognostic marker in GCs (data not shown). Remarkably, LGR5-positive GC patients had worse clinical outcomes than LGR5-negative GC patients ($P = 0.007$) (Fig. 4b), suggesting LGR5 is a poor prognostic marker when the

cases are restricted to the GCs with abnormally enhanced Wnt signaling.

Effects of LGR5 expression on the growth and migration of GC cells

As LGR5 positivity was shown to be a worse prognostic marker for GC patients with increased Wnt signaling, we figured that LGR5 could have a functional effect on cancer cell growth or migratory activity. LGR5 expression was examined in 15 GC cell lines, and most of the GC cell lines, except AGS and SNU-620, showed extremely low levels of LGR5 mRNA (Fig. 5a). No strong correlation was observed between LGR5 and ISC markers in GC cells (Fig. 5b). The LGR5-high group exhibited only slightly higher levels of ASCL2 than the LGR5-low group (Fig. 5b). Four GC cell lines with nuclear β -catenin expression (AGS, SNU-719, MKN-28, and MKN-74) and one without nuclear β -catenin expression (SNU-484) were transfected with empty vector or LGR5-expressing vector (Fig. 5c). The expression of LGR5 was assessed by RT-PCR analysis (Fig. 6a) and Western blot analysis (Fig. 6b) to demonstrate that LGR5 protein was expressed only in the AGS and SNU-484 cell lines, which were used for the functional experiments. The relative growth rate was

Table 1 Association between LGR5 expression and the clinicopathological characteristics

	Total	LGR5		<i>P</i>
		Negative	Positive	
Patients	603 (100 %)	561 (93 %)	42 (7 %)	
Age				
≥65 years	224 (37 %)	199 (89 %)	25 (11 %)	0.002 ^b
<65 years	379 (63 %)	362 (96 %)	17 (4 %)	
Sex				
Female	173 (29 %)	162 (94 %)	11 (6 %)	0.710 ^b
Male	430 (71 %)	399 (93 %)	31 (7 %)	
Histological differentiation				
Well	125 (21 %)	108 (86 %)	17 (14 %)	0.026 ^b
Moderate	240 (40 %)	223 (93 %)	17 (7 %)	
Poor	147 (24 %)	143 (97 %)	4 (3 %)	
Signet ring cell	62 (10 %)	60 (97 %)	2 (3 %)	
Papillary	15 (3 %)	13 (87 %)	2 (13 %)	
Other types	14 (2 %)	14 (100 %)	0 (0 %)	
Location				
Upper third	90 (15 %)	86 (96 %)	4 (4 %)	0.711 ^c
Middle third	226 (37 %)	208 (92 %)	18 (8 %)	
Lower third	277 (46 %)	258 (93 %)	19 (7 %)	
Whole	10 (2 %)	9 (90 %)	1 (10 %)	
Lymphatic invasion				
Negative	216 (36 %)	205 (95 %)	11 (5 %)	0.186 ^b
Positive	387 (64 %)	356 (92 %)	31 (8 %)	
Venous invasion				
Negative	499 (83 %)	468 (94 %)	31 (6 %)	0.133 ^b
Positive	104 (17 %)	93 (89 %)	11 (11 %)	
TNM stage ^a				
I	167 (27 %)	155 (93 %)	12 (7 %)	0.255 ^c
II	163 (27 %)	147 (90 %)	16 (10 %)	
III	213 (35 %)	198 (93 %)	15 (7 %)	
IV	66 (11 %)	61 (92 %)	5 (8 %)	
β-Catenin				
Nuclear stain	34 (6 %)	23 (68 %)	11 (32 %)	0.000 ^b
No nuclear stain	569 (94 %)	538 (95 %)	31 (5 %)	

^a American Joint Committee on Cancer's cancer staging manual, seventh edition^b Fisher's exact test^c Pearson's chi-square test

measured 4 days after transfection. However, there was no difference in the proliferation between cancer cells transfected with LGR5 and those transfected with control vector (Fig. 6c). In addition, LGR5 overexpression had no influence on the antiproliferative effect induced by 5-fluorouracil (100 µg/ml) (Fig. 6d). The wound healing assay revealed that LGR5 expression had no impact on the migration activity of GC cells either (Fig. 6e). These findings suggest that in vitro LGR5 overexpression alone is not enough to change the characteristics of GCs. Probably, additional signaling factors along with the Wnt/β-catenin

pathway are necessary to alter the biological behavior of GCs.

Discussion

LGR5 is the most promising stem cell marker in the normal gastric and intestinal mucosa. Using monoclonal antibodies against human LGR5 developed in the laboratory, Kemper et al. [13] provided evidence that LGR5 is also a marker for human colorectal cancers. However, the possibility of its

Fig. 3 Positive association of LGR5 expression with nuclear β -catenin positivity. A representative gastric cancer (a) with nuclear β -catenin expression (b) showed strong LGR5 expression (c), whereas

other gastric cancer (d) with membranous β -catenin expression (e) exhibited no LGR5 expression (f). Magnification $\times 400$

Fig. 4 Survival rate of gastric cancer patients with LGR5 expression. Kaplan–Meier analysis demonstrated that LGR5 positivity has no prognostic influence in gastric cancer patients ($n = 603$, $P = 0.560$).

However, for the gastric cancers with nuclear β -catenin expression, LGR5 expression was associated with poor survival ($n = 34$, $P = 0.007$). *Cum* cumulative

use as a stem cell marker in GCs has not been investigated yet even though LGR5 expression has been demonstrated through GC progression; normal gastric epithelium,

precancerous lesion, adenoma, and carcinoma [17, 25]. Because of the lack of commercially available antibodies that can recognize the extracellular domain and isolate

Fig. 5 Expression of LGR5 in the gastric cancer (GC) cell lines. **a** Fifteen GC cell lines were examined to determine the levels of endogenous LGR5, and five of them showed relatively higher LGR5 expression than the other ten GC cell lines. Asterisks indicate a group of LGR5-high cell lines. **b** GC cell lines with high LGR5 expression

were demonstrated to have higher levels of ASCL2 than those with low LGR5 expression. **c** Immunocytochemistry showed that the AGS, MKN-74, SNU-719, and MKN-28 cell lines are positive for nuclear β -catenin, whereas the SNU-484 cell line is negative. Magnification $\times 400$

LGR5-positive cells, we could not conduct isolation and subsequent functional experiments, which are considered the gold standard, to assess the potential of stem cell markers. Instead, in this study, we revealed the expression of LGR5 in a large number of human GC cases by RNA ISH and its significance for the prognosis to explore the biological implications of LGR5 in GC patients.

LGR5 mRNA was shown to be overexpressed in many types of cancers, such as colon cancer [16, 28], hepatocellular carcinoma [29], esophageal cancer [30], and basal cell carcinoma [31], compared with corresponding normal tissues. In addition, we previously demonstrated overexpression of LGR5 in gastric adenomas compared with normal gastric mucosa [25]. Unexpectedly, however, we found that the mean level of LGR5 in GCs was significantly lower than that in matched noncancerous gastric tissues. This result may be explained by the fact that noncancerous gastric tissues obtained from the same stomach of GC patients are highly likely to harbor pre-cancerous lesions, especially abundant foci of intestinal metaplasia. It is well known that intestinal-type GCs develop through a multistep process (chronic atrophic gastritis, intestinal metaplasia, and dysplasia), and most GCs are found in the background of intestinal metaplasia

[32]. Given the remarkable increase of LGR5 expression in intestinal metaplasia [25], variable degrees of intestinal metaplasia in noncancerous gastric mucosa could result in higher levels of LGR5 than in GCs. Although Yamanoi et al. [18] have reported upregulation of LGR5 in advanced GC tissues in comparison with noncancerous tissues, in which only 17 of 73 corresponding normal tissues were included for the comparison owing to the inadequate sample quality, which might have caused sampling bias. Also, the difference in the extent of intestinal metaplasia between study populations can lead to the discrepancy of the results in GCs.

By RNA ISH, we found that only 7 % of GCs expressed LGR5 mRNA. Notably, LGR5 expression was observed more frequently in older age (older than 65 years). Age differences in LGR5 positivity may be related to the fact that LGR5 expression is higher in intestinal-type GCs (well to moderately differentiated GCs) than in diffuse type GCs (poorly differentiated and signet ring cell type GCs) since diffuse-type GCs are commoner in the young age group [33]. It has been shown that LGR5 expression in gastric adenomas is positively correlated with nuclear β -catenin expression [25]. Likewise, we found that LGR5 positivity was higher in GCs with nuclear β -catenin expression. This close

Fig. 6 Effects of LGR5 expression on the growth and chemosensitivity of gastric cancer cells. **a** Reverse transcription PCR analysis showed increased LGR5 transcripts in all gastric cancer cells transfected with LGR5. **b** Western blot analysis confirmed that only the AGS and SNU-484 cell lines expressed a high level of LGR5 protein. **c** There was no difference in the growth rate between LGR5-

overexpressing and control cells. **d** No difference was observed in the relative cell growth 48 h after exposure to 5-fluorouracil (100 μ g/ml) between vector and LGR5-transfected cells. **e** Migration was evaluated by a wound healing assay, showing no difference in migration activity between control and LGR5 expression groups at 48 h after scratching

correlation between LGR5 and increased Wnt signaling was also reported in colon cancers [15, 34] as well as hepatocellular carcinomas [29], in which overexpression of LGR5 was observed in 87.5 % of hepatocellular carcinomas with mutation of β -catenin exon 3. These findings are in accordance with the fact that *LGR5* is one of the Wnt target genes and functions to augment canonical Wnt signals.

Most previous studies suggested that LGR5 expression is correlated with worse clinical outcomes. The prognostic impact of LGR5 has been most extensively studied in colorectal cancers, and recently two meta-analyses have concluded that LGR5 is related to the reduced overall survival in colorectal cancers [22, 35]. In addition, Nakata et al. [21] reported that LGR5 expression increased with

glioma progression and correlated with an adverse outcome, and Becker et al. [30] showed that high LGR5 expression was associated with worse survival in esophageal adenocarcinoma. For GCs, previous studies have reported inconsistent results. Xi et al. [24] showed that LGR5-positive patients had a significantly shorter survival time than LGR5-negative patients. On the other hand, Bauer et al. [36] suggested that *LGR5* mRNA expression was not a prognostic marker in GCs even though LGR5 expression increased in tumors after neoadjuvant chemotherapy. Our data show that LGR5 expression is not an overall prognostic marker for GCs, but it is a poor prognostic marker when restricted to GCs with nuclear β -catenin expression. Given that LGR5 functions to enhance Wnt signaling, this finding suggests the possibility that LGR5 expression contributes to aggressive behavior of GCs with an activated Wnt pathway.

Although LGR5 expression in colon cancers is usually believed to be associated with poor survival, some studies have reported conflicting results regarding the prognostic influence of LGR5. Ziskin et al. [14] and Takahashi et al. [37] have shown no link between LGR5 expression and overall survival of colon cancer patients. These two studies examined *LGR5* mRNA by RNA ISH or real time-PCR, whereas all the other studies reporting worse correlation of LGR5 with survival were based on the detection of LGR5 protein by immunohistochemistry. Thus, it seems that the difference in the method to detect LGR5 expression may be responsible for the discrepancy in the prognostic implications of LGR5. In fact, there is no reliable and commercially available antibody against LGR5 for use in immunohistochemistry, whereas it was clearly proved that LGR5-expressing cells on human FFPE samples can be specifically marked by RNA ISH [25]. Therefore, we believe that measuring mRNA either by RNA ISH or by RT-PCR would currently be more appropriate to evaluate the clinical significance of LGR5 with human specimens.

To determine whether LGR5 is functionally involved in the progression of GCs with aberrant Wnt activation, we performed in vitro growth and migration assays with GC cell lines with nuclear β -catenin expression. Unfortunately, however, we could not find any growth benefit with LGR5 expression in GC cells. One possible explanation for this result is the lack of an appropriate microenvironment in the in vitro experiments because Wnt signaling is also regulated by interactions between tumor cells and stromal cells. For instance, myofibroblasts expressing a high level of hepatocyte growth factor can activate Wnt signaling and even restore the stem cell phenotype in more differentiated tumor cells [38]. The Hedgehog and Notch pathways are also known to be deeply involved in the cancer stem cell properties [4]. Thus, our results suggest that LGR5 overexpression alone is not sufficient to recapitulate the

molecular characteristics of LGR5-positive cells in GC. Maybe more complex and coordinated signaling pathways are required to fully activate the Wnt signaling and in turn affect the biological behavior of GC cells. We cannot rule out the possibility that LGR5 expression may be more of an indicator representing a stem cell phenotype than a functional factor in maintaining the stem cell properties. If so, induced expression of LGR5 in GCs is not an appropriate way to evaluate the significance of LGR5 as a prognostic marker. As mentioned earlier, isolation of LGR5-expressing cells from GC specimens and their subsequent molecular characterization of is required to help unravel the significance of LGR5 as a prognostic marker in GCs.

In summary, LGR5 expression of GCs was lower than that of the corresponding noncancerous gastric tissues. Only 7 % of GCs examined were positive for LGR5, which was correlated with older age, well-differentiated histologic type, and nuclear β -catenin expression. Although LGR5 was not a prognostic marker for all GC cases, it was associated with worse survival for GCs with nuclear β -catenin expression, suggesting a functional role of LGR5 in cancer progression of GCs with abnormally enhanced Wnt signaling. However, in vitro assays provided no evidence of a survival benefit of LGR5, demanding further studies on the biological roles of LGR5 to facilitate the investigation of its potential as a candidate marker for the targeted therapy of GCs.

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