Distribution of *Lgr5*-positive cancer cells in intramucosal gastric signet-ring cell carcinoma

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A short running title: Lgr5-positive gastric cancer cells

Abstract

Leucine-rich repeat-containing G-protein-coupled receptor 5 (*Lgr5*) is a putative intestinal stem cell marker. *Lgr5* is also expressed in various tumors. To identify *Lgr5* expression in mucosal gastric signet-ring cell carcinoma (SRCC) and analyzed its pathological characteristics. We investigated *Lgr5* expression in 35 patient samples of intramucosal gastric SRCC using RNAscope®, a newly developed RNA in situ hybridization technique. *Lgr5* expression in individual tumor cells was scored semi-quantitatively on a scale (H-score) from 0 to 400. Ki67 was also examined by immunohistochemistry, with a linear arrangement of Ki67-expressing cells present in 20 of 35 cases. This area of Ki67-expressing cells was topographically divided into three areas: upper, middle, and lower. All cases with linear Ki67 expression patterns also had *Lgr5*-positive cells arranged in a linear fashion in the lower area—which was distinct from the area of high Ki67 expression. The rate of Ki67 positivity in *Lgr5*-positive cells was significantly lower than that of *Lgr5*-negative cells in areas of high Ki67 expression. These characteristics suggest that *Lgr5*-positive cells may represent cancer stem cells in SRCC. Intramucosal SRCC may therefore contain stem cells expressing *Lgr5* in the lower area of the lamina propria, akin to normal gastric pyloric mucosa.

Keywords: stem cell marker; *Leucine-rich repeat-containing G-protein-coupled receptor 5*; signet-ring cell carcinoma; RNA in situ hybridization ; Ki67

Leucine-rich repeat-containing G-protein-coupled receptor 5 (*Lgr5*) is a putative intestinal stem cell marker[1, 2]. Hair follicles and brain tissues express *Lgr5*, and *Lgr5* is also over-expressed in various tumors, including those of the liver and colon[3-5]. *Lgr5* expression in gastric carcinoma has been reported; however, the clinicopathological characteristics of this expression remain poorly defined[6]. The difficulties surrounding immunostaining of *Lgr5* have contributed to the lack of knowledge in this area.

Gastric carcinoma has many subtypes, though few studies have focused on *Lgr5* expression in gastric signet-ring cell carcinoma (SRCC). Early gastric carcinoma is defined as invasive carcinoma confined to mucosa and/or submucosa, with or without lymph node metastases, and of variable tumor size[7]. We have investigated *Lgr5* expression in intramucosal gastric SRCC—an early gastric carcinoma—and analyzed several pathological characteristics using a newly described, highly sensitive RNA in situ hybridization technique (RNAscope®). This bright-field in situ hybridization-based assay enables visualization of target cellular RNA species in formalin-fixed paraffin-embedded tissue sections. Recent investigations have demonstrated the presence of *Lgr5*-positive cells at the base of pyloric glands. Although no *Lgr5*-positive cells were detected at the fundic glands, gastric intestinal metaplasia show *Lgr5*-positive cells at the base of intestinal metaplastic glands[8].

The search for a specific cancer stem cell (CSC) marker in gastric carcinoma is ongoing. Presently, *Lgr5* appears to be the most promising gastric carcinoma CSC marker[8]. While several studies have employed RNAscope® to identify gastric CSC markers[8], the distribution of *Lgr5* expression based on histological type has not been clarified. Intramucosal gastric SRCC is a special carcinoma with organoid differentiation [9, 10], which is the stratification of tumor cells that mimic normal gastric mucosa. Typical signet ring cells occur in the upper and lower layers, while the middle layer contains smaller tumor cells. An understanding of *Lgr5* distribution in intramucosal gastric SRCC may assist elucidation of histological regulation in this form of cancer, and could assist in future CSC-targeted therapies.

MATERIALS AND METHODS

Patients and materials

We identified 35 cases of intramucosal gastric SRCC at Shinshu University Hospital, Matsumoto, Japan from January 2010–July 2014, and evaluated their clinicopathological features. This study was approved by the Ethics Committee of Shinshu University, Japan (no. 2345).

Histopathology and immunohistochemistry

All specimens were fixed in 20% formaldehyde and embedded in paraffin. Tumor blocks with sufficient tissue were selected to prepare a tissue microarray (TMA). The most representative region of the tumor was selected based on the morphology of the hematoxylin and eosin (HE)-stained slide. Tissue cores were punched out from each donor tumor block using thin-walled 3-mm stainless steel needles (Azumaya Medical Instruments Inc., Tokyo, Japan), and cores were arrayed in a recipient paraffin block. Serial sections of 4-μm thickness were cut from these blocks and stained with HE, immunostained with anti-CD10 (1:100; Leica Biosystems, Nussloch, Germany), anti-MUC2 (1:100; Novocastra, Newcastle upon Tyne, United Kingdom), anti-MUC5AC (1:100; Novocastra), anti-MUC6 (1:100; Novocastra), anti-Pepsinogen I (1:100, AbD Serotec, Kidlington, UK), or anti-Ki67(1:50, Dako, Glostrup, Denmark) antibodies. Positive staining was recorded when more than 10% of the area returned a positive signal, as per guidelines established previously[11].

***Lgr5* RNA in situ hybridization**

Detection of *Lgr5* mRNA was performed using the RNAscope® kit (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer's instructions using unstained sample tissue slides. Briefly, tissue sections were pretreated by heating and protease application prior to hybridization with an *Lgr5*-specific probe. The detailed procedure is described in an earlier publication[12]. Brown punctate dots present in the nucleus and/or cytoplasm indicated positive staining. *Lgr5* expression was quantified according to the five-grade scoring system recommended by the manufacturer (0: no staining, 1: 1–3 dots/cell, 2: 4–10 dots/cell, 3: > 10 dots/cell, 4: > 15 dots/cell with > 10% of dots in clusters). The H-score was calculated as: (% of grade 1 cells × 1) + (% of grade 2 cells × 2) + (% of grade 3 cells × 3) + (% of grade 4 cells × 4). The overall H-score for each patient was calculated based on the H-score per high-power field (400× magnification). Furthermore, a cell with 1 or more dots was regarded as *Lgr5*-positive.

Combination *Lgr5* RNA in situ hybridization and Ki67 immunohistochemistry

Intramucosal gastric SRCC cases with a linear arrangement of Ki67-expressing cells in the lamina propria were selected for further analysis with a combination of *Lgr5* RNA in situ hybridization and Ki67 immunohistochemistry. Briefly, tissues were

immunostained with anti-Ki67 antibody (1:50; Dako) followed by in situ detection of *Lgr5* mRNA using the RNAscope® kit, as described above.

Samples from cases with a linear arrangement of Ki67-expressing cells in the lamina propria were divided into three areas—upper, middle, and lower. The H-score for each of these areas was then determined for each patient. Expression of Ki67 was determined by examining the same region used to calculate the H-score. Additionally, the number of cells staining double-positive for both *Lgr5* and Ki67 was measured in all three areas in samples with a high H-score.

Statistical Analysis

Statistical analysis was performed using JMP version 10 (SAS Institute Japan, Tokyo, Japan). Spearman's rank correlation coefficient analysis was used to assess correlation. The Wilcoxon rank sum test was applied to assess the statistical significance of associations between H-score of *Lgr5* and Ki67 expression. A P value < 0.05 was considered significant.

RESULTS

Clinicopathological factors do not correlate with *Lgr5* RNA expression

TMA cores included all layers of the mucosa, which was sufficient to evaluate histopathological characteristics. Clinicopathological data of SRCC cases are listed in Table 1. Expression of *Lgr5* was detected in all cases of SRCC. Furthermore, all SRCC cases were classified as mixed type. No cases possessed detectable pepsinogen I expression. Table 2 summarizes the correlation between *Lgr5* H-score and clinicopathological characteristics of patients with SRCC. The *Lgr5* H-score did not correlate with age, tumor size, MUC2 expression, MUC5AC expression, MUC6 expression, or CD10 expression.

Ki67 and *Lgr5* expression patterns in SRCC

Values for *Lgr5* H-score varied among the cases. Twenty cases exhibited a linear arrangement of Ki67-expressing cells in the lamina propria (Fig. 1A), and all of these cases also possessed a high density of linearly arranged dots indicating *Lgr5* expression in the lamina propria (Fig. 1B). Furthermore, cases with a layered structure of Ki67 staining had organoid differentiation as determined by HE staining (Fig. 1C and D). Of

the remaining cases with non-linear Ki67 staining patterns, 13 had scattered regions of *Lgr5*-positive cells and 2 had diffuse regions of *Lgr5*-positive cells.

Areas of high *Lgr5* H-score have lower expression of Ki67

Mean H-scores for *Lgr5* staining in the upper, middle, and lower areas of the lamina propria were 17.5 (10–32.5), 27.5 (16.3–40), and 47.5 (25.5–86.3), respectively. The *Lgr5* H-score was significantly higher in the lower area compared with either the upper or middle areas ($P < 0.001$; Fig. 2A). Additionally, the *Lgr5* H-score for the middle area was significantly higher than that of the upper area ($P < 0.001$; Fig. 2A). Conversely, Ki67 staining was detected in cells of the upper, middle, and lower areas at rates of 15% (10–35%), 47.5% (31.3–60%), and 12.5 (5–23.8%), respectively. The percentage of Ki67-positive cells was significantly higher in the middle area compared with the upper and lower areas ($P < 0.001$; Fig. 2B). There was no significant difference in the rate of Ki67-positivity between the upper and lower areas ($P = 0.410$; Fig. 2B). A combination of *Lgr5* RNA in situ hybridization and Ki67 immunohistochemistry was performed in samples from 20 cases that exhibited a layer-like structure of Ki67 staining in the lamina propria (Fig. 3A and B). Four other cases were not evaluated as sufficient

specimen material was not available. Within the high-Ki67 staining areas of the 16 cases analyzed, Ki67-positivity was significantly reduced in *Lgr5*-positive cells (38.1%, range: 30.5–64.4) compared with *Lgr5*-negative cells (55.5%, range: 38.6–76.1; $P < 0.001$; Fig. 4).

DISCUSSION

To our knowledge, this is the first study characterizing the distribution and proliferative activity of *Lgr5*-positive cells in intramucosal gastric SRCC. Organoid differentiation of intramucosal gastric SRCC has been previously described[9]. Moreover, a proliferative zone with a layer structure exists in intramucosal gastric SRCC with organoid differentiation[10, 13, 14]. All samples with a layer structure of Ki67 staining had organoid differentiation and a layer structure of *Lgr5* expression in our study. The highest levels of Ki67 and *Lgr5* expression were observed in respectively different layers. This pattern of a high proliferation zone distinct from the high *Lgr5*-expression zone is the same as for normal pyloric mucosa[15]. Therefore, these results suggest that *Lgr5*-positive cells may include a CSC population in SRCC.

There was no correlation between *Lgr5* expression and the clinicopathological variables examined in our study. In contrast, previous studies have identified a positive correlation between *Lgr5* expression and depth of tumor invasion, lymph node metastasis, and distant metastasis in invasive gastric carcinoma[16, 17]. Another study has reported a positive association between *Lgr5* expression and age, tumor differentiation, Lauren type, and TNM stage[16]. Furthermore, recent investigations have demonstrated that patients expressing *Lgr5* have a worse prognosis than those that do not [18, 19]. To the best of our knowledge, no prior reports examining associations between clinicopathological variables and *Lgr5* expression in early gastric carcinoma exist. Our present findings suggest that *Lgr5* expression is not associated with prognostic factors for intramucosal gastric SRCC.

While the *Lgr5* H-score was low, all intramucosal gastric SRCC cases possessed *Lgr5*-positive cell populations. This suggests that SRCC CSCs may be considered *Lgr5*-positive cells. SRCC can be classified into subtypes including gastric, mixed, and intestinal types[20]. Intestinal gastric carcinoma expresses *Lgr5*[8], so intestinal-type SRCC may possess an intestinal phenotype with *Lgr5*-expression. Gastric-type SRCC with pyloric features also possesses *Lgr5* expression, and pyloric glands contain *Lgr5*-positive stem cells[15]. However, gastric-type SRCC with fundic features and

expression of pepsinogen I and II has been reported in a minority of cases[21]. These tumors are believed to arise from chief cells of the fundic gland. Because chief cells do not express *Lgr5*, SRCC originating from chief cells may have another type of CSC[15]. Further investigation of SRCC of chief cell origin is warranted to determine whether other SRCC CSC populations exist.

CSCs are considered to have a low mitotic activity similar to normal stem cells, and our present findings are consistent with this. However, the mitotic rates of CSCs have yet to be identified in most solid cancers. Lu et al. reported that *Lgr5*-expressing cells had high mitotic activity in colon cancer[22]. *Lgr5* is believed to be a target of Wnt signaling, so activation of the Wnt signaling pathway via mutation of the adenomatous polyposis coli gene during development of colon carcinoma may lead to *Lgr5* expression[23]. Moreover, the mitotic ability of CSCs likely depends on multiple factors, including cancer type, gene mutation, and the microenvironment.

Few studies have examined *Lgr5* expression in the progression of gastric SRCC. In our study, patient samples lacking organoid differentiation had varied distributions of *Lgr5*-positive cells. SRCC without organoid differentiation may therefore be irregular. In invasive gastric carcinoma, *Lgr5* expression was observed at both the tumor center and the invasive front[6]. However, this previous report is based on

immunohistochemistry data, and detection of Lgr5 protein by immunohistochemistry is unreliable. We previously reported the presence of Lgr5 in the pyloric mucosa of gastric cancer patients using immunohistochemistry [24]. Furthermore, Lgr5-positive hematopoietic cells were also detected at this site[24]. However, this staining may represent a non-specific positive reaction, as other reports have described non-specific staining of parietal, stromal, and endothelial cells following immunohistochemical staining for *Lgr5*[6, 25]. Such non-specific staining may reflect the weak expression of *Lgr5* in the gastric mucosa and related carcinoma. Therefore, investigation of *Lgr5* distribution in gastric carcinoma based on histological type should be repeated using RNAscope®.

Our study is limited by its small sample size, which includes only patients who underwent surgery at our hospital. Furthermore, the TMA examined small samples that may not necessarily be representative of entire tumors. However, the histological classification of all samples was SRCC. Moreover, TMA cores contained all layers of mucosa, which enabled SRCC evaluation. Nevertheless, our present results should be verified using larger cohorts.

The SRCC CSC may be an *Lgr5*-positive cell. The layered structure of *Lgr5* expression we have identified is a new characteristic of SRCC, which can be included

alongside organoid differentiation as a marker of this type of tumor. *Lgr5* expression may therefore serve as a target for therapy in gastric carcinoma, especially SRCC. Further studies using larger sample sizes are necessary to determine the precise role of *Lgr5* expression in the stomach and its utility as a prognostic indicator in gastric tumors.

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TABLES

Table 1 Clinicopathological characteristics of 35 SRCC patients

Variable	Value	Number of patients
Median age (years)		
	≤61	18
	>61	17
Sex		
	Male	18
	Female	17
Tumor Location		
	Upper	1
	Middle	26
	Lower	8
Median tumor size (mm)		
	≤37	18
	>37	17
Macroscopic type		
	2a	2
	2b	2
	2c	31
Lgr5 expression		
	Positive	35
	Negative	0
Median H-score		
	≤40	20
	>40	15
MUC2		
	Positive	19
	Negative	16
MUC5AC		
	Positive	33

	Negative	2
MUC6		
	Positive	27
	Negative	8
CD10		
	Positive	34
	Negative	1
Phenotypic classification		
	Intestinal phenotype	0
	Gastric phenotype	0
	Mixed phenotype	35
	Unclassified phenotype	0
Pepsinogen I		
	Positive	0
	Negative	35

SRCC: signet-ring cell carcinoma

Table 2 Correlation between clinicopathological characteristics and *Lgr5* H-score

Variable	*R*	*P*
Age	0.1881	0.2783
Size	0.0659	0.7067
MUC2	0.2498	0.1479
MUC5AC	0.11	0.5293
MUC6	0.067	0.7022
CD10	0.0719	0.6816

r = Spearman's rank correlation coefficient;

P = *P*-value

FIGURE LEGENDS

Figure 1

HE, immunohistochemical staining for Ki67, and *Lgr5* RNA in situ hybridization in SRCC samples. A. Staining for Ki67-positive cells. B. Identification of *Lgr5*-positive cells. Original magnification for B–D: 50×. C. SRCC cells were detected in the lamina propria (Original magnification: 100×). D. The layered structure is composed of an upper and a lower layer of signet ring cells and a middle layer of small round cells.

Figure 2

Lgr5 H-scores and Ki67-positivity in SRCC. A. *Lgr5* H-scores for upper, middle, and lower regions. B. Ki67-positivity H-scores for upper, middle, and lower regions. Scores are expressed as minimum, 25th and 75th (percentiles), and maximum.

*$P < 0.001$ (Wilcoxon rank sum test).

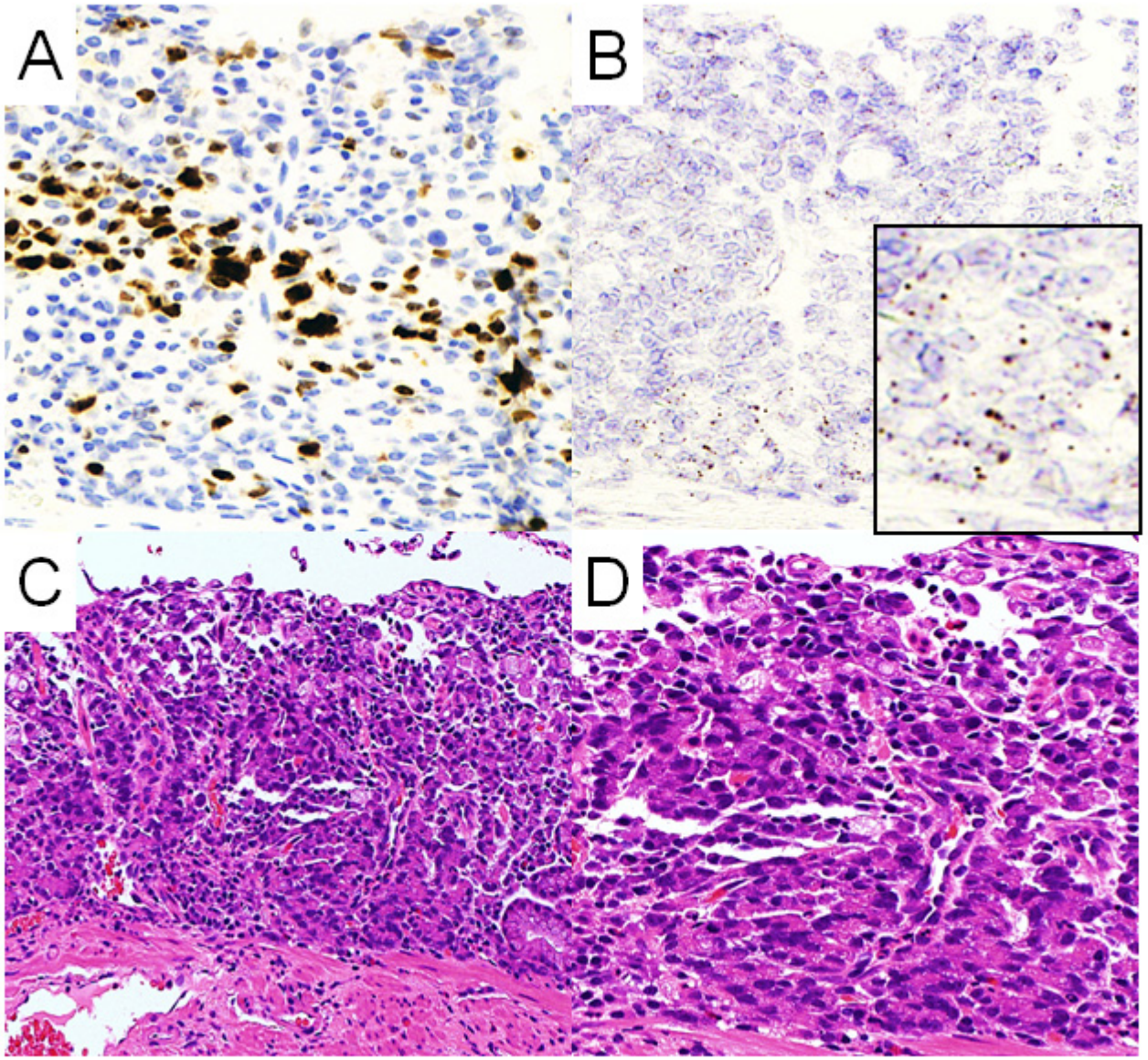
Figure 3

Combination of *Lgr5* RNA in situ hybridization with Ki67 immunohistochemistry for assessment of SRCC samples. A. Ki67 (red) and *Lgr5* (brown) staining of cells in SRCC tissue sections (100×). B. Double-positive cells are indicated (arrows; 200×).

Figure 4

Ki67 positivity by immunohistochemistry in combination with *Lgr5* RNA in situ hybridization. Ki67 positivity in *Lgr5*-positive and -negative regions was compared. Scores are expressed as minimum, 25th and 75th (percentiles), and maximum.

*$P < 0.001$ (Wilcoxon rank sum test).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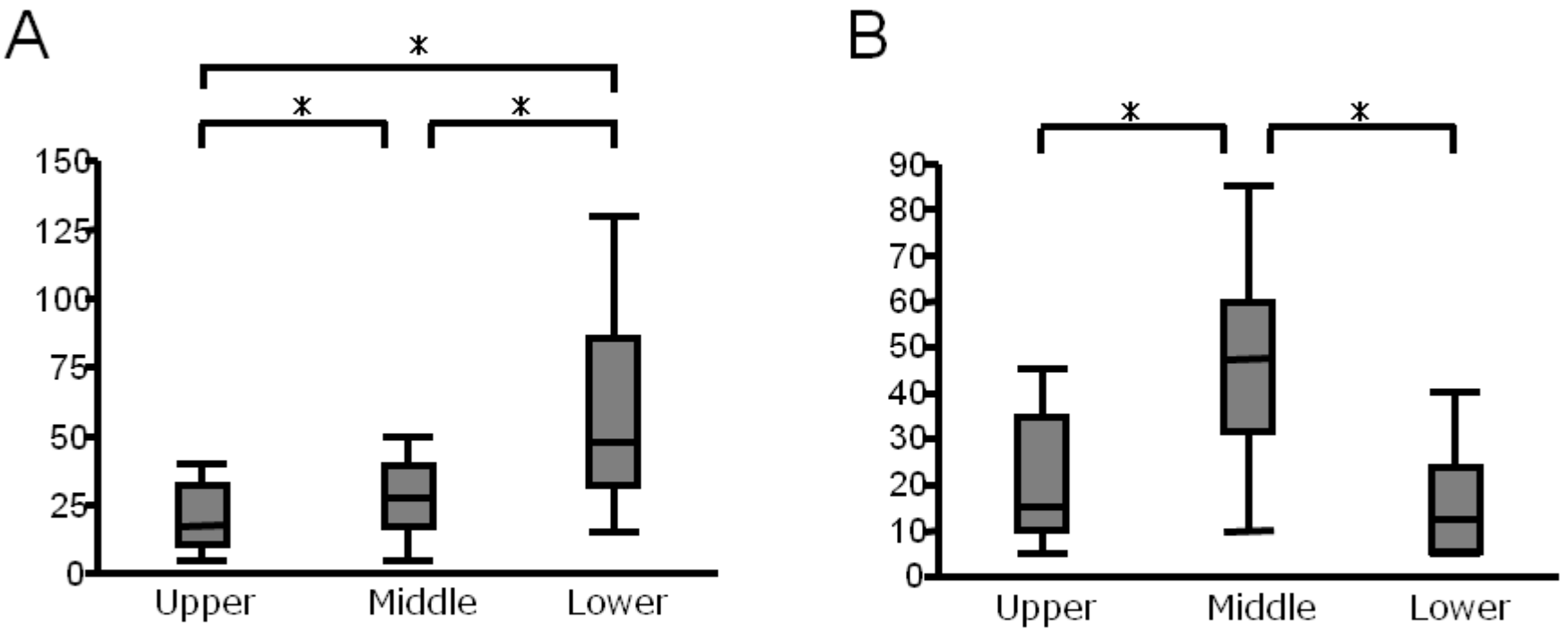
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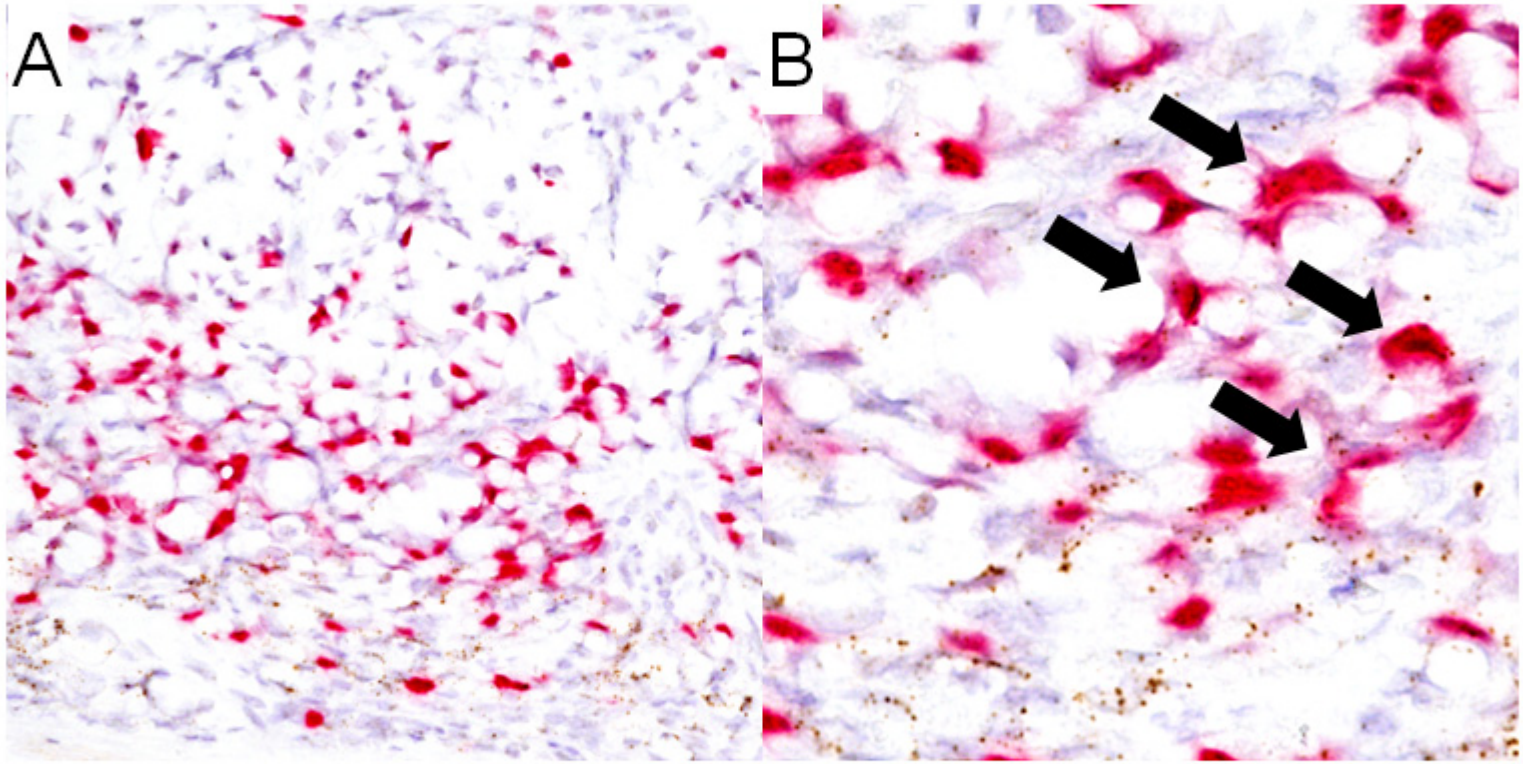
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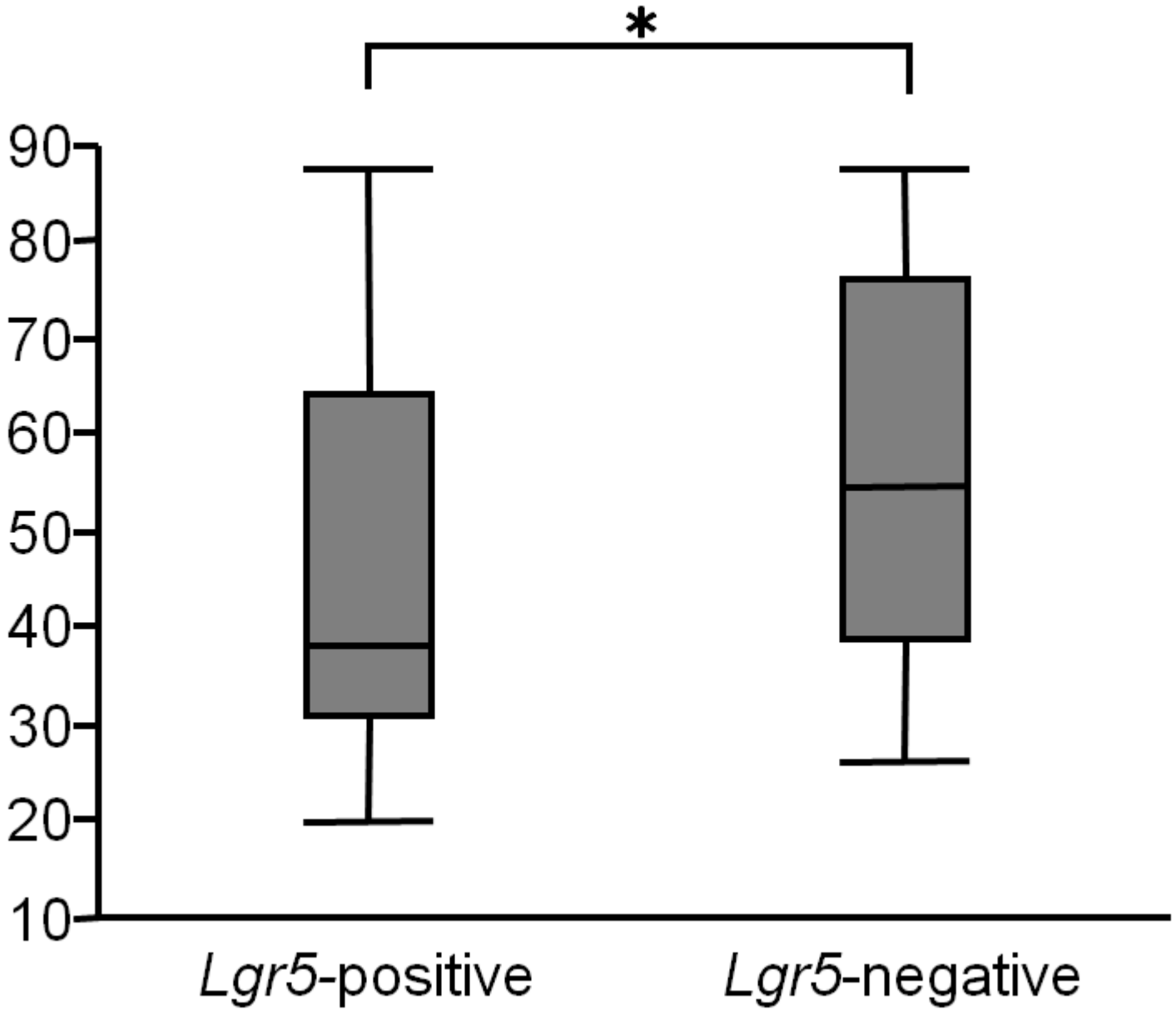
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Lgr5-positive
Lgr5-negative