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Original Contribution

Correlation of clinicopathological features and *LGR5* expression in colon adenocarcinoma

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ABSTRACT

Colon cancer stem cells (CSCs) are closely related to tumorigenesis and treatment response, and *LGR5* is currently the most robust and reliable CSC marker in colorectal cancer (CRC). However, *LGR5* expression in CRC tumor budding (TB) is not well understood. We examined the clinicopathological and prognostic significance of *LGR5* in CRC TB. *LGR5* expression was evaluated by RNAscope, a newly developed RNA in situ hybridization technique, using a tissue microarray consisting of 55 patient samples of TB in colon adenocarcinoma (CA) selected from the medical archives at our hospital. Patients were stratified into negative and positive *LGR5* expression groups. Tumor-infiltrating lymphocytes (TILs) and histological grade were lower in the *LGR5*-positive group compared with the *LGR5*-negative group ($P = .0407$ and $P = .0436$, respectively). There was no significant difference in overall survival between the *LGR5*-positive group and the *LGR5*-negative group (log-rank test, $P = .6931$). *LGR5* expression did not remain a predictor of prognosis in univariate analysis (OR = 0.84, 95% CI: 0.33–2.02, $P = .6928$). *LGR5* expression may be affected by TILs, which have been demonstrated to be associated with worse prognosis in the budding area of CA and is an important potential marker of prognosis.

1. Introduction

Colorectal cancer (CRC) is the second most common cause of cancer-related death worldwide [1]. Although early diagnosis and immediate surgery can cure patients with CRC, adjuvant chemotherapy is selected for high-risk CRC (stages II and III). However, stages II and III CRC are reported to recur in 30% and 40% of patients, respectively, despite adjuvant chemotherapy [2]. Since CRC recurrence causes a decrease in survival rate, development of a new treatment method is required.

The significance of cancer stem cells (CSCs) in cancer has been emphasized in recent years. CSCs comprise a small percentage of the total cancer tissue but are involved in self-renewal and metastasis and are resistant to chemotherapy [3,4]. LGR5 was identified by the lineage tracing method as the most promising stem cell marker in the colorectum and is considered a CSC marker in CRC [5-8]. It has also been reported as a CSC marker in several other tumors including those of the stomach [9-11], liver [12], and esophagus [13]. Several studies have analyzed *LGR5* expression and prognosis [14,15]. There are several reports that high *LGR5* expression is associated with poor prognosis but this is controversial [16,17].

The invasion front, especially tumor budding (TB) in CRC, is the forefront of tumor cell attack and host side defense and provides important prognostic information [18,19]. The clinicopathological and prognostic significance of *LGR5* was analyzed in CRC TB using RNAscope, a newly described RNA in situ hybridization technique.

2. Materials and methods

2.1. Patients

This study enrolled 55 patients with colon adenocarcinoma (CA)

Abbreviation: CRC, colorectal cancer; CSC, cancer stem cell; LGR5, leucine-rich repeat-containing G-protein-coupled receptor 5; TB, tumor budding; CA, colon adenocarcinoma

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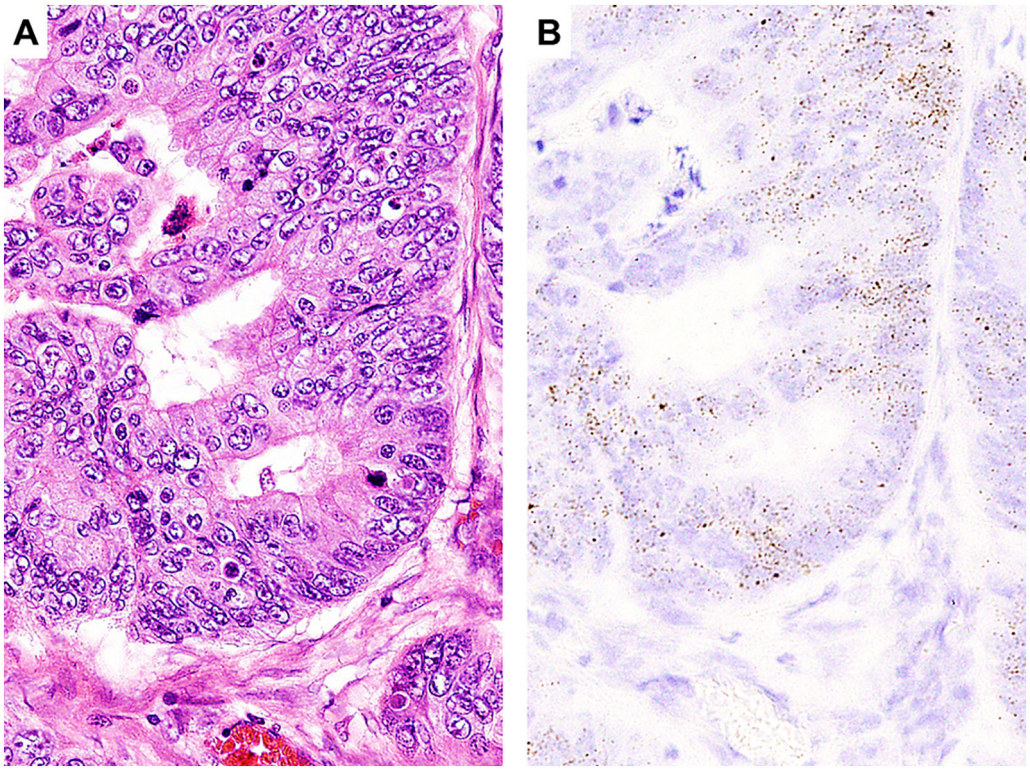

Fig. 1. Representative features of *LGR5* expression in CA (A and B). *LGR5* expression was prominent in the gland-forming region recognized as low-grade CA. (A, HE; B, *LGR5* RNAscope).

with TB who underwent surgical resection between 2010 and 2012 at Shinshu University School of Medicine, Matsumoto, Japan. Patients were followed up for at least 1 year. Clinicopathological data such as patient age and sex, pathological differentiation, inflammatory cell infiltration, vascular invasion, and TNM classification were obtained by retrospective review of medical charts and pathological records. Clinical stage and tumor differentiation were determined using the 8th edition of Union International Cancer Control (UICC) TNM staging system. Histological features of all specimens were confirmed by two pathologists (T.U. and H.O.). Overall survival (OS) was defined as the interval between the date of surgical resection and date of death or last follow up. This study was performed in accordance with the current ethical guidelines of the Declaration of Helsinki and the requirements of the Institutional Review Board of Shinshu University School of Medicine (approval No. 4088).

2.2. 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 TB, defined as an area with a single cell or a detached group of tumor cells consisting of five cells or less, was selected based on the morphology of the hematoxylin and eosin (H&E) 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 H&E. TB was graded into Bd1 (0–4 buds), Bd2 (5–9 buds) and Bd3 (≥10 buds) [20]. Furthermore, TB grade was categorized into low grade (Bd1 and Bd2) and high grade (Bd3). The score of inflammatory cell infiltration (tumor infiltrating lymphocytes, TILs) was measured in the budding area. The TIL score was assessed using a four-titer score and recorded as following; none: 0, mild: 1, moderate: 2, and marked: 3 [21]. Furthermore, TIL score was categorized into low grade (score 0 and 1) and high grade (2 and 3).

2.3. LGR5 RNA in situ hybridization

Detection of *LGR5* mRNA was performed using an RNAscope kit (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer's instructions using unstained sample tissue slides. Briefly, tissue sections were pretreated with heating and protease application prior to hybridization with an *LGR5*-specific probe. The detailed procedure is described in a previous report [22]. Brown punctate dots present in the nucleus and/or cytoplasm indicated positive staining. *LGR5* expression was quantified according to the 5-grade scoring system recommended by the manufacturer (no staining; 0, 1–3 dots/cell; 1+, 4–10 dots/cell; 2+, 10–15 dots/cell; 3+, and > 15 dots/cell; 4+) under a 20× objective lens (Olympus BX51). Furthermore, *LGR5* mRNA expression was categorized into negative expression (grade 0 and 1+) and positive expression (2+, 3+, and 4+). We analyzed the relationship between *LGR5* expression and clinicopathological data and prognosis in CA patients, with particular regard to OS rate.

2.4. Statistical analysis

For clinicopathological characteristics, categorical variables were expressed as a number. Pearson's chi-squared tests were adopted to test for differences between subgroups of patients. The OS rates of CA patients were calculated using the Kaplan-Meier method, and differences in those rates were compared by the log-rank test. The univariate and multivariate analyses for prognostic factors were performed using a Cox proportional hazard regression model. A P-value of < 0.05 was considered significant. All statistical analyses were performed using JMP Statistics software version 13 (JMP, Tokyo, Japan).

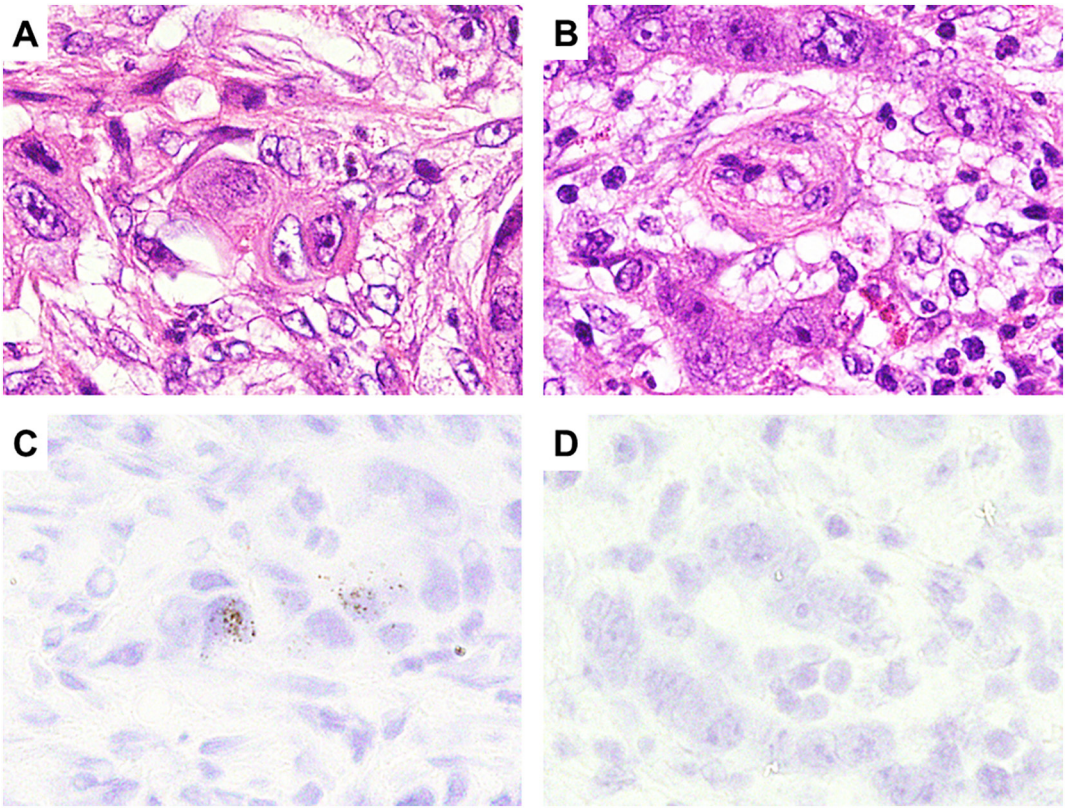

Fig. 2. *LGR5* expression in TB. Representative features in TB with low-grade TILs (A) and high-grade TILs (B). In TB with low-grade TILs, high levels of *LGR5* expression was observed (C). However, in TB with high-grade TILs, *LGR5* expression was negative (D). (A and B, HE; C and D, *LGR5* RNAscope).

3. Results

3.1. LGR5 expression in CA

In all cases, some *LGR5*-positive dots were detected in tumor cells, and there were a wide range of *LGR5*-positive cells. Fig. 1 shows representative images of *LGR5* expression in CA. Expression of *LGR5* was prominent in the gland-forming region. In TB, 35 cases showed some *LGR5*-positive dots over a wide range, but there were no positive findings in 20 cases. Twenty-six cases were classified into an *LGR5*-positive group (Fig. 2A, C) and 29 cases were classified into an *LGR5*-negative group (Fig. 2B, D).

3.2. LGR5 expression and clinicopathological characteristics in CA

The clinicopathological characteristics of patients with CA are described in Table 1. In the *LGR5*-positive group, TILs occurred in lower numbers than in the *LGR5*-negative group ($P = .0407$). In the *LGR5*-positive group, histological grade was lower than that of the *LGR5*-negative group ($P = .0436$). There was no significant difference between the *LGR*5-positive group and the *LGR5*-negative group in terms of age, sex, vascular invasion, or TNM stage.

3.3. Prognostic value of LGR5 in CA

We assessed the prognostic value of *LGR5* expression in CA by Kaplan-Meier analysis and the log-rank test. The median OS rate for the study patients was 1839 (range: 949–2379) days. There was no significant difference in OS between CA cases in the *LGR5*-positive group (median OS, 1988 [range, 972.25–2545.5] days) and the *LGR5*-negative group (median OS, 1837 [range; 887–2188] days) (log rank test, $P = .6931$).

LGR5 expression did not remain a predictor of prognosis in univariate analysis (OR = 0.84, 95% CI: 0.33–2.02, $P = .6928$) (Table 2).

Table 1
LGR5 expression and clinicopathological characteristics in CA.

Factors	n	*LGR5* expression		*P*-value
		Positive ($n = 26$)	Negative ($n = 29$)	
Age				0.5039
> 70 years	27	14	13	
≤70 years	28	12	16	
Sex				0.1416
Male	29	11	18	
Female	26	15	11	
TILs				0.0407
High	27	9	18	
Low	28	17	11	
Histological grade				0.0436
High	29	10	19	
Low	26	16	10	
Vascular invasion				0.9444
High	32	15	17	
Low	23	11	12	
Tumor budding grade				0.6093
High	10	4	6	
Low	45	22	23	
TNM stage				0.4394
I–II	22	9	13	
III–IV	33	17	16	

4. Discussion

Although there is no direct involvement of *LGR5* expression on OS in TB, the correlation between *LGR5* expression and TILs may indicate that high *LGR5* expression confers a poor prognosis, considering that

Table 2
Univariate analyses for prognostic factors of CA.

Factors	Univariate analysis	
	OR (95% CI)	*P*-value
Age: > 70 years vs ≤70 years	2.59 (1.06–6.92)	0.0369
Sex: male vs. female	2.84 (1.13–8.03)	0.0251
Histological grade: low vs. high	0.52 (0.20–8.03)	0.1574
TIL: low grade vs. high grade	3.46 (1.34–10.66)	0.0095
Vascular invasion: absent vs. present	0.72 (0.27–1.76)	0.4771
Tumor budding grade: low vs. high	0.41 (0.16–1.16)	0.0896
TNM stage: I–II vs. III–IV	0.54 (0.19–1.35)	0.1928
LGR5 expression: positive vs. negative	0.84 (0.33–2.02)	0.6928

low-grade TIL is involved in poor prognosis [23]. It is reported that TILs are related to microsatellite instability-high (MSI-H) CRC [24]. MSI-H CRC may be classified into one category of the disease called hypermutated type. Therefore, it may be necessary to increase the number of cases and examine *LGR5* expression in the presence or absence of TILs. Evaluation of TB in CRC is performed using various techniques. Although TB grade showed no significant association with OS in our study, there are several reports on the number of tumor cells and the degree of differentiation in TB, as well as the relationship between the type of TB and prognosis [25,26].

The expression of various markers in TB has been analyzed and there have been several reports of CSC marker expression in recent years. In the TB the decreased expression of CD44, which is known to be a representative stem cell marker, has been reported to indicate a poor prognosis [27]. Although another stem cell marker, CD133, exhibits negative or weak positivity in TB, no association with clinicopathological data has been reported [28,29].

LGR5 is recognized not only as the most promising stem cell marker in the colorectum but also as a CSC marker in CA [5-8]. Several studies on *LGR5* expression and clinicopathological analysis in CA have been reported, but there are few reports on TB [30,31]. Zheng et al. reported that TB is not related to *LGR5* expression and distant metastasis [30]. Moreover, the relationship between distant metastasis and *LGR5* expression is controversial in other tissues [7,30,32]. *LGR5* has been reported to be associated with markers that promote epithelial-mesenchymal transition (EMT), which promotes cancer metastasis [33]. Although the expression of many genes related to EMT has been reported in tumor cells of TB [34], Jang et al. reported that attenuation of *LGR5* expression in TB correlates with promotion of EMT [31]. In our study, there was no significant difference between *LGR5* expression and prognosis. However, in *LGR5*-positive cases, the promotion of EMT was involved in metastasis and may result in poor prognosis.

In TB, the *LGR5*-positive group with significantly low-grade CA might have been affected by the *LGR5* expression levels in low-grade CA tissue with gland formation. In our present study, *LGR5* expression was more commonly identified in low-grade CA with gland formation. The relationship between tumor grade and *LGR5* expression level is controversial [14,30-32,35]. Many of these reports used immunohistochemistry to evaluate *LGR5*. However, in recent years, RNA in situ hybridization has been reported to reveal strong expression of *LGR5* in low-grade CA with gland formation [31]. This difference may be because the *LGR5* expression site differs between the two methods. However, because the reliability of immunohistochemistry for *LGR5* is questionable [31], RNA in situ hybridization may be more reliable.

Declining TIL numbers in the *LGR5*-positive group may be a protective function of stem cells. *LGR5* expression may be affected by TILs in the budding area of CA. There is no report on the relationship between *LGR5* and TILs despite there being reports on stem cell markers in other cancer types. The expression of ALDH1, which is considered to be a CSC marker in breast cancer, is high in TIL-positive cases [36]. Since ALDH1 is also an enzyme involved in detoxification, its function differs from that of LGR5 [37]. Therefore, the reversal in the expression levels of LGR5 and ALDH1 in TILs may be due to differences in carcinomas and in the function of CSC markers.

A limitation of our study is that only *LGR5* mRNA expression was evaluated and thus the functional analysis of LGR5 protein expression in cultured cells is warranted. It may also be necessary to analyze a larger number of cases in a long-term follow-up cohort study.

In conclusion, this study showed that high *LGR5* expression in TB, which is more frequent in low-histological grade CA, is involved in low-grade TIL, which has been demonstrated to be associated with poor prognosis, and thus *LGR5* expression in TB may have important implications in CSC-targeted therapy.

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Declaration of competing interest

None declared.

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