LGR5 expression is associated with prognosis in poorly differentiated gastric adenocarcinoma

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Short title: *LGR5* in poorly differentiated gastric adenocarcinoma

Abstract

Background: Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5*)* is an important cancer stem cell marker in gastric cancer. However, no detailed studies are available on LGR5 expression in poorly differentiated gastric adenocarcinoma (PD-AC). Therefore, we investigated the relationship between LGR5 expression and clinicopathological data in PD-AC.

Methods: *LGR5* expression was identified in 41 PD-AC cases using RNAscope, which is a highly sensitive RNA *in situ* hybridization method. Epstein–Barr virus (EBV) infection was also detected by EBV *in situ* hybridization.

Results: In PD-AC, *LGR5* expression was identified in 38 of 41 cases, and 17 cases were identified as *LGR5* positive. The frequency of EBV positivity tended to be higher in the *LGR5*-negative group than in the *LGR5*-positive group (P=0.0764). Furthermore, the frequency of vascular invasion tended to be higher in the *LGR5*-positive group than in the *LGR5*-negative group (P=0.0764). A significant difference was found in overall survival (OS) between PD-AC cases in the *LGR5*-positive group and *LGR5*-negative group (log-rank test, P=0.0108). The Cox proportional hazard regression model revealed that the *LGR5*-negative group (HR=0.29; 95% CI: 0.11–0.74; P=0.01) showed independently better OS for PD-AC.

Conclusions: The correlation between *LGR5* positivity and poor prognosis in PD-AC may be applicable to target therapy for *LGR5* and prognostic markers. Further study is warranted.

Keywords: leucine-rich repeat-containing G-protein-coupled receptor 5; poorly differentiated gastric adenocarcinoma; RNA *in* situ hybridization

Background

Although the frequency of gastric cancer occurrence is decreasing, many individuals develop the disease. Gastric cancer is the fifth most frequent cancer and the third leading cause of cancer death [1]. For gastric cancer, various treatment methods, including surgery, radiation, and chemotherapy, have been performed. Among them, many recent studies have been conducted on cancer stem cells (CSCs), which are present in tumor tissue, and anti-CSC therapy targeting them has high expectations [2]. CSCs are resistant to radiation and chemotherapy, and residual CSCs contribute to tumor re-growth. Therefore, anti-CSC therapy effective for CSC should be performed in combination with existing methods such as chemotherapy and radiotherapy [3]. Some reports have investigated gastric cancer CSCs, which typically express CD44, CD133, and Musashi-1 [4]. Additionally, leucine-rich repeat-containing G-protein-coupled receptor 5 (*LGR5*) may be a robust CSC marker in gastric cancer identified by a new method [5] [6].

The components of poorly differentiated cancer tissues have a great impact on prognosis. In gastric cancer, poorly differentiated adenocarcinoma (PD-AC) has a poor prognosis [7], but the underlying reasons remain unclear. The expression of CSC markers in poorly differentiated gastric cancer, especially *LGR5*, has not been reported. Therefore, we report the clinicopathological relationship between *LGR5* marker expression and prognosis.

Methods

Patients and materials

We identified 91 cases of PD-AC at Shinshu University Hospital, Matsumoto, Japan from 2008 to 2018 and evaluated their clinicopathological features. Stage II and III cases were selected from all cases, and 41 cases were candidates for analysis. This study was approved by the Ethics Committee of Shinshu University, Japan (no. 4088).

Histopathology, immunohistochemical staining, and evaluation

Paraffin blocks containing sufficient tumor for analysis, fixed with 8% formaldehyde, were prepared for TMA by extracting a core with a diameter of 3 mm from each case, as well as HE staining. Additionally, the TMA was subjected to immunostaining using antibodies against the following mismatch repair proteins (MMRP): MLH1 (ES05; mouse monoclonal; dilution 1:50), PMS2 (EP51; rabbit monoclonal; dilution 1:40), MSH2 (FE11; mouse monoclonal; dilution 1:50), or MSH6 (EP49; rabbit monoclonal; dilution 1:50; Agilent Technologies, Santa Clara, CA, USA) as described previously [8]. As reported in our previous paper, the staining results were scored as positive when a nuclear staining pattern was observed. If at least one of the four antibodies did not show expression, MMR protein deficiency was indicated. The TIL score was assessed using a four-tier score and was recorded as follows: none: 0; mild: 1; moderate: 2; and marked: 3 [9]. Furthermore, the TIL score was

categorized as low grade (score 0 and 1) and high grade (2 and 3).

EBER *in situ* hybridization

The EBER *in situ* hybridization assay was performed on TMA block sections. EBV was identified using EBER probes (ISH iVIEW Blue detection kit; Ventana Medical Systems Inc., Oro Valley, AZ, USA).

***LGR5* RNA in situ hybridization**

LGR5 mRNA detection was performed using the RNAscope® kit (Advanced Cell Diagnostics, Hayward, CA, USA) as described previously [8]. Additionally, a four-step evaluation method that we reported previously was used [8]. Furthermore, *LGR5* mRNA expression was categorized as negative expression (grades 0, 1+, 2+, and 3+) and positive expression (4+). We analyzed the relationship between *LGR5* expression and the clinicopathological data and prognosis in patients with PD-AC, particularly regarding overall survival (OS).

Statistical analysis

Chi-squared test was applied to assess the statistical significance. A P-value < 0.05 was considered to be statistically significant. The OS rates of PD-AC patients were calculated using the Kaplan–

Meier method, and differences were compared using the log-rank test. Univariate and multivariate analyses for prognostic factors were performed using the Cox proportional hazard regression model. A P-value <0.05 was considered to be statistically significant. Statistical analysis was performed using JMP version 13 (SAS Institute Japan, Tokyo, Japan).

Results

LGR5 expression in PD-AC

In PD-AC, 38 of 41 cases had *LGR5* expression. Among them, 17 cases were identified as *LGR5* positive (Fig. 1A and 1B). Moreover, *LGR5* expression was completely absent in three cases (Fig. 1D and 1E). *LGR5* expression varied from diffuse to scattered.

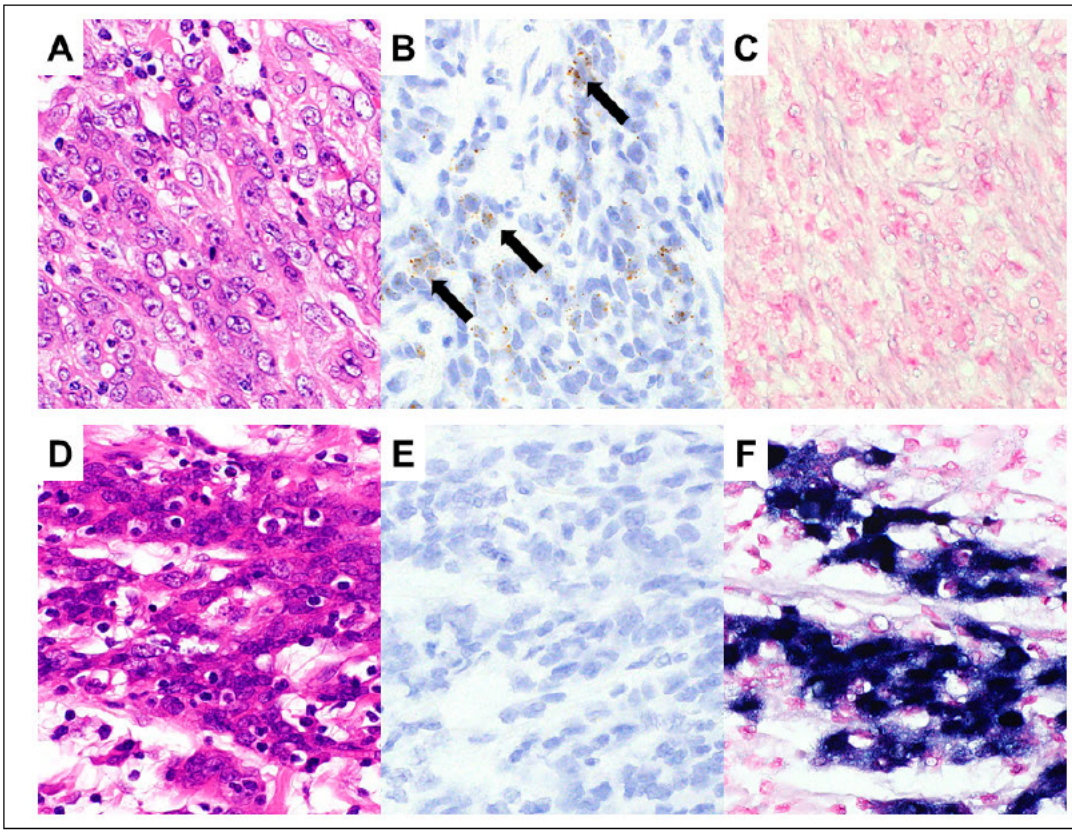

Figure 1 Representative images of *LGR5* and EBV

Representative features in *LGR5*-positive (A and B) and *LGR5*-negative (D and E) cases. *LGR5*-positive cases show EBV negativity (C). *LGR5*-negative cases show EBV positivity (F). (A and D, HE; B and E, *LGR5*; C and F, EBV).

Relationship between *LGR5* expression and clinicopathological characteristics

EBV expression was negative in most cases (Fig. 1C). Although there were few EBV-positive cases in PD-AC (Fig. 1F), all were identified as *LGR5* negative. We showed the *LGR5* and clinicopathologic data in Table 1. EBV-positive PD-AC tended to be higher in the *LGR5*-negative group than in the *LGR5*-positive group (P=0.0764). The frequency of vascular invasion tended to be higher in the *LGR5*-positive group than in the *LGR5*-negative group (P=0.0764). No significant

difference was found between the *LGR5*-positive group and *LGR5*-negative group regarding TILs, MSI, histological subtype, or TNM stage.

Table 1 *LGR5* expression and clinicopathological characteristics in PD-AC

Factors	n	*LGR-5* expression		P value
		Positive (n=17)	Negative (n=24)	
Age				0.0472
>74 years	19	11	8	
≤ 74 years	22	6	16	
Sex				0.0498
Male	24	13	11	
Female	17	4	13	
EBV				0.0764
Positive	4	0	4	
Negative	37	17	20	
Vascular invasion				0.0764
Present	37	17	20	
Absent	4	0	4	
TIL				0.283
High	25	9	16	
Low	16	8	8	
MSI				0.2176
Present	19	10	9	
Absent	21	7	14	
Differentiated-type				0.2921
Solid-type 1	8	2	6	
Non-solid-type 2	33	15	18	
TNM stage				0.9382
II	19	8	11	
III	22	9	13	

Prognostic value of *LGR5* in PD-AC

The prognostic value of *LGR5* expression in PD-AC was analyzed by Kaplan–Meier analysis and the log-rank test (Fig. 2). The median OS for the study patients was 1,146 (range; 635.5–1718) days. A significant difference was found in OS between PD-AC cases in the *LGR5*-positive group (median OS: 756 (range; 154.5–1306.5) days) and *LGR5*-negative group (median OS: 1338 (range; 922.75–2022.75) days) (log-rank test, P=0.0108).

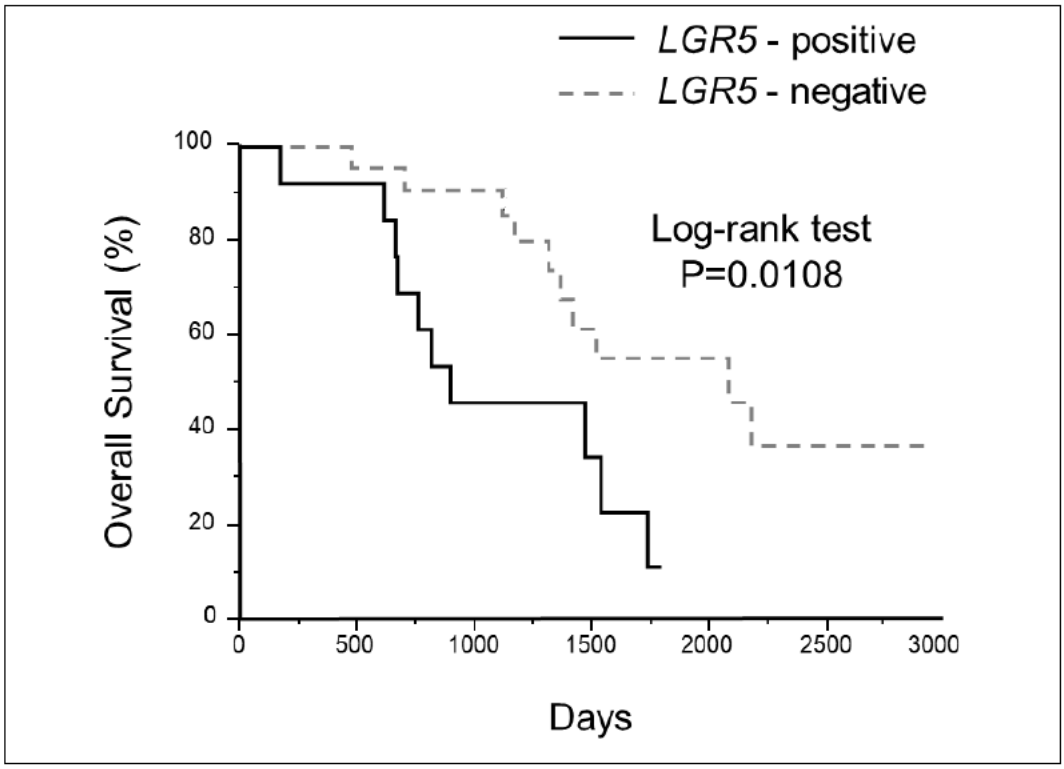

Figure 2 Prognostic value of *LGR5* in CA by Kaplan–Meier analysis.

A significant difference was found in OS between PD-AC cases in the *LGR5*-positive group (median OS; 756 (range; 154.5–1306.5) days) and *LGR5*-negative group (median OS; 1338 (range; 922.75–2022.75) days; log-rank test, P=0.0108).

We evaluated the relationship between clinicopathological factors and *LGR5* expression regarding OS using a Cox proportional hazard regression model (Table 2), which revealed that the *LGR5*-negative group (HR = 0.29; 95% CI: 0.11–0.74; P= 0.01) had independently better OS for PD-AC.

Table 2 Univariate and multivariate analyses for prognostic factors of PD-AC

Factors	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Age: >74 years vs ≤ 74 years	1.49 (0.57–3.69)	0.3986		
Sex: Male vs Female	0.65 (0.26–1.69)	0.3596		
Vascular invasion: Present vs Absent	3.07 (0.64–55.22)	0.1936		
TIL: Low vs High	1.94 (0.77–4.86)	0.1575		
MSI: Present vs Absent	2.07 (0.79–5.39)	0.1338		
TNM stage: II vs III	0.39 (0.15–0.97)	0.0425	0.36 (0.13–0.89)	0.026
LGR5: Positive vs Negative	3.18 (1.24–8.39)	0.0161	3.48 (1.36–9.22)	0.01

Discussion

LGR5 is an independent prognostic factor in Stages II and III of PD-AC. Although PD-AC has a poor prognosis [7], the related factors are not well understood. PD-AC has solid and non-solid subtypes. The prognosis of non-solid with fibrosis is poor [10]. In our study, most PD-AC cases were non-solid, but no clear difference was shown in the prognosis of both non-solid and solid subtypes. *LGR5* is also a promising gastric cancer CSC marker, and high *LGR5* expression in the

poor prognosis group may suggest involvement of CSCs in the prognosis. Therefore, *LGR5* may be a therapeutic target in PD-AC and may improve PD-AC prognosis.

The migration ability and EMT are increased in poorly differentiated gastric cancer [11]. The cause of a poor prognosis at high *LGR5* expression may be related to the histological features of poorly differentiated cancer represented by migration ability acquisition and EMT-related protein expression and *LGR5* expression. Cancer cell migration is known to affect prognosis in gastric cancer[11]. Additionally, *LGR5* expression, although not in the stomach, is related to migration ability and EMT [12]. In our study, the correlation between vascular invasion and high *LGR5* expression may support an association of *LGR5* expression with EMT. High expression of *LGR5* and EMT was reported to be correlated in gastric cancer [13]. Furthermore, Zhang et al. reported that *RSPO2*, a ligand of *LGR5*, promotes EMT in gastric cancer cells by activating WNT/β-catenin signaling via *LGR5* [14]. Therefore, elucidation of the relationship between *LGR5* and *RSPO2* in PD-AC may lead to the development of new therapeutic methods and an improved prognosis for PD-AC.

Although some reports have indicated that high *LGR5* expression is associated with a poor prognosis[15] [16], others have reported that high *LGR5* expression correlates with a good prognosis in RNAscope studies, which are considered to have high reliability [17] [18]. The expression of *LGR5* in the tumors of various organs has been widely discussed; most of them are the results of

studies evaluated by immunostaining. The results of immunostaining may be controversial and uncertain. We pointed out that high *LGR5* expression might be a poor prognostic factor in breast cancer by RNAscope examination [19]. Further studies are needed regarding the prognostic impact of *LGR5* RNA expression.

Several reports have investigated *LGR5* expression in gastric cancer. One has indicated no significant difference in the OS of gastric cancer when examined using RNAscope [20]. Although evaluated by immunostaining, *LGR5* overexpression was reported to be significantly associated with an increased risk of death in gastric cancer patients in a review of *LGR5* expression in gastric cancer [21]. In addition, Bu *et al*. reported that *LGR5* expression is associated with a favorable prognosis, although it was limited to Stages I and II [22]. Furthermore, *LGR5* expression was associated with a high stage and lymph node metastasis [20]. Although no association was found between high *LGR5* expression and the histological type, as pointed out in a previous review [20], Xi *et al.* reported that high *LGR5* expression is associated with poorly differentiated cancer [23]. However, Bu *et al.* reported that *LGR5* is highly expressed in well-differentiated cancer [22]. Additionally, in studies using RNAscope, high *LGR5* expression is correlated with well-differentiated cancer [24]. However, there is no comparison between *LGR5* expression and prognosis in PD-AC using RNAscope, and the association between high *LGR5* expression and poor prognosis in poorly differentiated cancer is a new finding.

The tendency for low *LGR5* expression in EBV expression may be a feature of EBV-associated gastric cancer. EBV-associated gastric cancer is recognized as a distinct type of gastric cancer, according to a novel molecular pathological classification [25] and is recognized as a cancer with a good prognosis [26]. The Cancer Genome Atlas Research Network reports that gastric cancer is divided into four types [25]. Therefore, it is necessary to analyze *LGR5* expression in each, hoping that further knowledge will be accumulated in the future.

Our study possesses some limitations. This study had a relatively small sample size, which may have led to unreliable estimates. *LGR5* expression and migration must be investigated in cultured cells; additionally, *LGR5* expression must be analyzed in EBV-infected cells.

Conclusions

The involvement of LGR5 expression in the prognosis of poorly differentiated gastric cancer may be applicable to targeted therapy for LGR5 and prognostic markers. Further study is desired.

List of abbreviations

CSC: cancer stem cell; LGR5: leucine-rich repeat-containing G-protein-coupled receptor 5; PD-AC: poorly differentiated adenocarcinoma; TMA: tissue microarray; TILs: tumor-infiltrating lymphocytes; OS: overall survival.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shinshu University School of Medicine (Approval Code: 4088). The requirement of informed consent was waived. and an opt-out method was used due to the retrospective design of the study. The investigation was conducted in compliance with the Helsinki Declaration.

Consent for publication

Not applicable.

Availability of data and materials

All the data generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TE participated in the design of the study, performed the pathological analysis, and drafted the manuscript. TU, SK, and MI helped with the pathological analysis. TU performed the statistical analysis. TN and YK conducted immunohistochemistry. TE and US examined the clinical data of cases. HO and TU revised the draft critically for important intellectual content.

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