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Correlation of clinicopathological features and leucine-rich repeat-containing G-protein-coupled receptor 5 expression in pancreatic ductal adenocarcinoma

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer. Previous studies have established leucine-rich repeat-containing G-protein-coupled receptor 5 (*LGR5*) as a cancer stem cell marker in gastrointestinal cancers. However, few reports have examined *LGR5* in PDAC. Here we examined *LGR5* expression and its clinicopathological significance in PDAC. We evaluated *LGR5* expression in 78 PDAC patients who underwent surgical resection in our institution using RNAscope, a newly described RNA *in situ* hybridization technique. All 78 PDAC cases expressed *LGR5* in cancer tissues, and *LGR5* expression was prominent in the gland-forming part. *LGR5* expression was significantly higher in patients with low histological grade (G1–G2) ($p < 0.001$) and early clinical stage ($p = 0.004$). Univariate analysis showed that low *LGR5* expression ($p = 0.034$) was significantly associated with worse overall survival. However, *LGR5* expression did not remain a predictor of prognosis in multivariate analysis ($p = 0.639$). All PDAC cases showed *LGR5* expression to varying degrees, indicating *LGR5* might be a cancer stem cell marker of PDAC, as in gastrointestinal cancer. Reduced *LGR5* expression in tumor cells was associated with worse prognosis in PDAC. Further studies are required to elucidate the relationship between tumor progression and *LGR5* expression in PDAC.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer death worldwide [1]. Pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer, shows extremely poor prognosis and the only potentially curative treatment is surgical resection. However, the prognosis of PDAC patients who have undergone resection still remains extremely poor, with a 5-year survival rate ranging from 10% to 18% [2–5]. Moreover, PDAC is usually diagnosed in the inoperable advanced stage, and effective therapies for these patients are lacking. Given the limitations of conventional therapies for PDAC, the identification of therapeutic targets and prognostic markers for PDAC to improve survival outcome is critical.

Tumors contain a small subpopulation of cancer stem cells (CSCs) that possess self-renewal capacity and drive tumorigenicity [6,7]. CSCs are hypothesized to be exclusively responsible for tumor progression, and studies have demonstrated that CSCs are highly resistant to chemotherapy and radiotherapy [8]. Therefore, the development of therapies targeting CSCs is a reasonable approach to improve patient outcome. Several studies have attempted to identify CSCs in PDAC, but the precise role of stem cell populations in the progression of PDAC remains unclear [9,10].

Leucine-rich repeat containing G-protein-coupled receptor 5 (*LGR5*) is the most promising and established stem cell marker in the gastrointestinal tract [11]. *LGR5* expression has been observed and its clinicopathological significance has been demonstrated in various cancers, including cancers of the colon [12–14], stomach [15–17], liver [18] and esophagus [19]. The prognostic significance of *LGR5* expression has been intensively investigated, especially in colorectal cancer, and many reports have shown that *LGR5* expression is related to poor prognosis [20,21]. The significance of *LGR5* expression in PDAC, however, remains unknown. A few studies have indicated the presence of LGR5-

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positive cells in PDAC by immunohistochemical analysis [15,22]. No reports have yet assessed the clinical significance of *LGR5* expression in PDAC, including survival outcome.

In this study, we examined the association between *LGR5* expression and prognosis and clinicopathological features in PDAC using RNAscope, a new RNA *in situ* hybridization technique.

2. Materials and methods

2.1. Patients

This study enrolled 78 patients with PDAC who underwent surgical resection between 2005 and 2013 at Shinshu University School of Medicine, Matsumoto, Japan. Clinicopathological data such as patient age and gender, tumor location, adjuvant chemotherapy, pathological differentiation, tumor size, lymphatic metastasis and TNM classification were obtained by retrospectively reviewing medical charts and pathological records. Clinical stage and tumor differentiation were determined using the 8th edition of the Union International Cancer Control TNM staging system and the 4th edition of the World Health Organization classification. 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 the last follow up. This study was performed in accordance with the current ethical guidelines of the Declaration of Helsinki and was conducted in accordance with the requirements of the Institutional Review Board of Shinshu University School of Medicine (approval No. 4020).

2.2. Histopathology

All tumor specimens were fixed in 20% formaldehyde and embedded in paraffin. Tumor blocks with sufficient tissue were selected for a tissue microarray. 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 the blocks and stained with HE.

2.3. LGR5 RNA in situ hybridization

Detection of *LGR5* mRNA was performed on unstained sample tissue slides with the RNAscope kit (Advanced Cell Diagnostics, Hayward, CA, USA), according to the manufacturer's instructions. Briefly, tissue sections were pretreated by heating and protease application prior to hybridization with a *LGR5*-specific probe. The detailed procedure has been previously described [23]. The standard positive control (Mm-PPIB, ACD-313902) and negative control (DapB, ACD-310043) probes were used to ensure interpretable results. Brown punctate dots in the nucleus and/or cytoplasm indicated positive staining. *LGR5* expression was quantified according to the 5-grade scoring system (no staining, grade 0; 1–3 dots/cell, grade 1; 4–10 dots/cell, grade 2; 10–15 dots/cell, grade 3; > 15 dots/cell, grade 4). 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 H-score for each case was calculated as the mean score of three representative areas in high-power fields (400× magnification). Cases with an H-score higher than the mean H-score were categorized in the high *LGR5* expression group, while cases with H-scores lower than the mean were categorized in the low *LGR5* expression group. We analyzed the relationship between the degree of H-score and clinicopathological data and prognosis in PDAC patients, especially the 5-year survival rate. We also evaluated *LGR5* expression in non-cancerous tissues, including five healthy pancreas cases and five obstructive pancreatitis cases.

2.4. Statistical analysis

In clinicopathological characteristics, categorical variables were expressed as number and percentages. Pearson's chi-squared tests were adopted to test for differences between subgroups of patients. The survival rates of PDAC 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*-

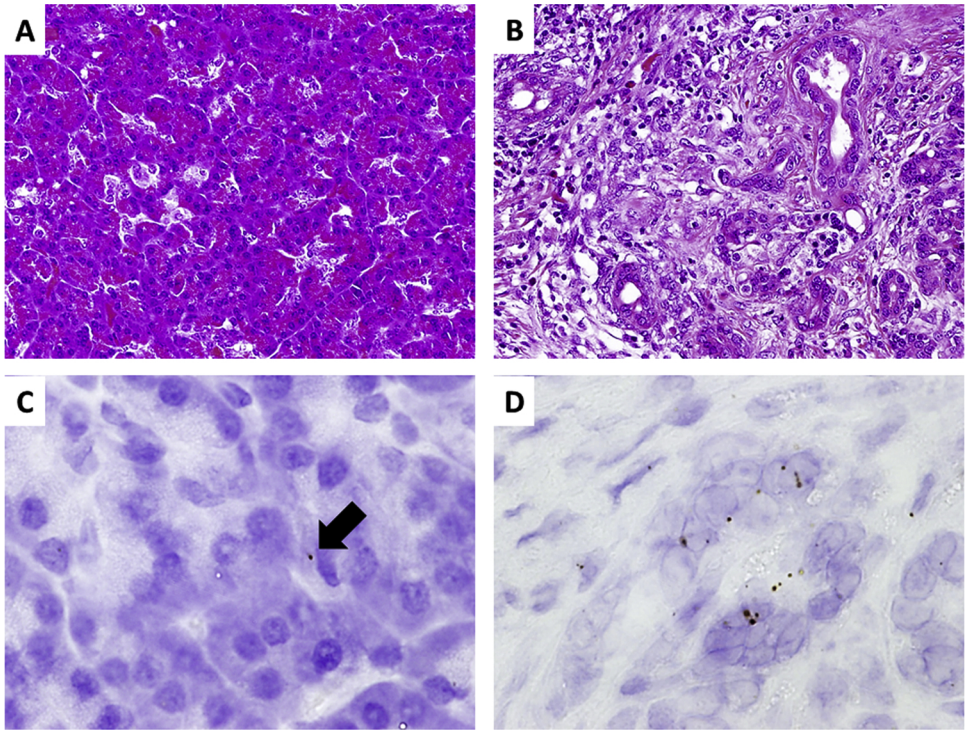

Fig. 1. *LGR5* expression in normal pancreas tissue and pancreatitis tissue. Representative HE images of normal pancreas tissue (A) and pancreatitis tissue (B). Normal pancreas tissue showed nearly negative *LGR5* staining, but a small number of positive dots (arrow) was observed in a very small number of parts of intercalated and intralobular ducts (C). In pancreatitis tissue, more positive *LGR5* expression in the same site as normal tissue was observed compared with normal tissue (D). (A and B, HE, 200x; C and D, *LGR5* RNAscope, 400x).

value < 0.05 was considered to be significant. All statistical analyses were performed using the JMP Statistics software version 13 (JMP, Tokyo, Japan).

3. Results

3.1. LGR5 expression in normal pancreas tissue and pancreatitis tissue

We first investigated the expression of *LGR5* in normal pancreas tissue and obstructive pancreatitis tissue that were obtained from surrounding tissue resected from PDAC cases. Representative images are shown in Fig. 1. In normal pancreas tissue, *LGR5* staining was almost negative, but a weak positive staining was observed in a very small number of parts of intercalated and intralobular ducts. The mean *LGR5* H-score in healthy pancreas cases was 2 (range, 1–4). In contrast, in obstructive pancreatitis tissue, more *LGR5*-positive findings in the same site as normal tissue were clearly observed compared with normal tissue. The mean *LGR5* H-score in obstructive pancreatitis cases was 13 (range, 6–18).

3.2. LGR5 expression in PDAC

In all 78 PDAC cases, *LGR5*-positive dots were detected in tumor cells, with a wide range of *LGR5*-positive cell staining. Fig. 2 shows representative figures of *LGR5* expression in PDAC. Expression of *LGR5* was prominent in the gland-forming part. The mean *LGR5* H-score in PDAC was 41 (range, 2–159).

3.3. LGR5 expression and clinicopathological characteristics in PDAC

The clinicopathological characteristics of the PDAC patients are listed in Table 1. The median age of the 78 PDAC patients was 69 years (range, 35–88). The clinical stages were as follows: 54 cases of early stage (stage I: 22 cases, stage II: 32 cases) and 15 cases of advanced stage (stage III: 13 cases, stage IV: 2 cases). The histological grades were as follows: 52 low grade cases (Grade 1: 29 cases, Grade 2: 23 cases) and 26 high grade cases (Grade 3: 26 cases, Grade 4: 0 cases). Sixty-six patients (85%) were treated with adjuvant chemotherapy and no patients received neoadjuvant chemotherapy. We categorized patients into high *LGR5* expression and low *LGR5* expression groups based on the mean H-score as described in the Methods. Low *LGR5* expression was detected in 46/78 (59%) patients, while high *LGR5* expression was detected in 32/78 (41%) patients. The expression of *LGR5* was significantly higher in low histological grade (G1–G2) ($p < 0.001$) and early clinical stage ($p = 0.004$). There was no significant difference between high and low *LGR5* expression in terms of sex, age, location of tumor, tumor size or lymph node metastasis. Fig. 3 is a box-whisker plot of *LGR5* H score in each histological grade. The H score decreased as the histological grade increased.

Table 1
LGR5 expression and clinicopathological characteristics in PDAC patients.

		LGR5 expression		
Factors	n	High (n = 32)	Low (n = 46)	P value
Age				0.815
≥65 years	50	21	29	
< 65 years	28	11	17	
Sex				0.981
Male	44	18	26	
Female	34	14	20	
Location of tumor				0.315
Head	51	23	28	
Body-tail	27	9	18	
Histological grade				< 0.001
Low (G1-2)	52	31	21	
High (G3)	26	1	25	
Tumor size				0.500
T1-2	63	27	36	
T3-4	15	5	10	
Nodal metastasis				0.283
Negative	24	12	12	
Positive	54	20	34	
TNM stage				0.004
I-II	54	28	26	
III-IV	24	4	20	

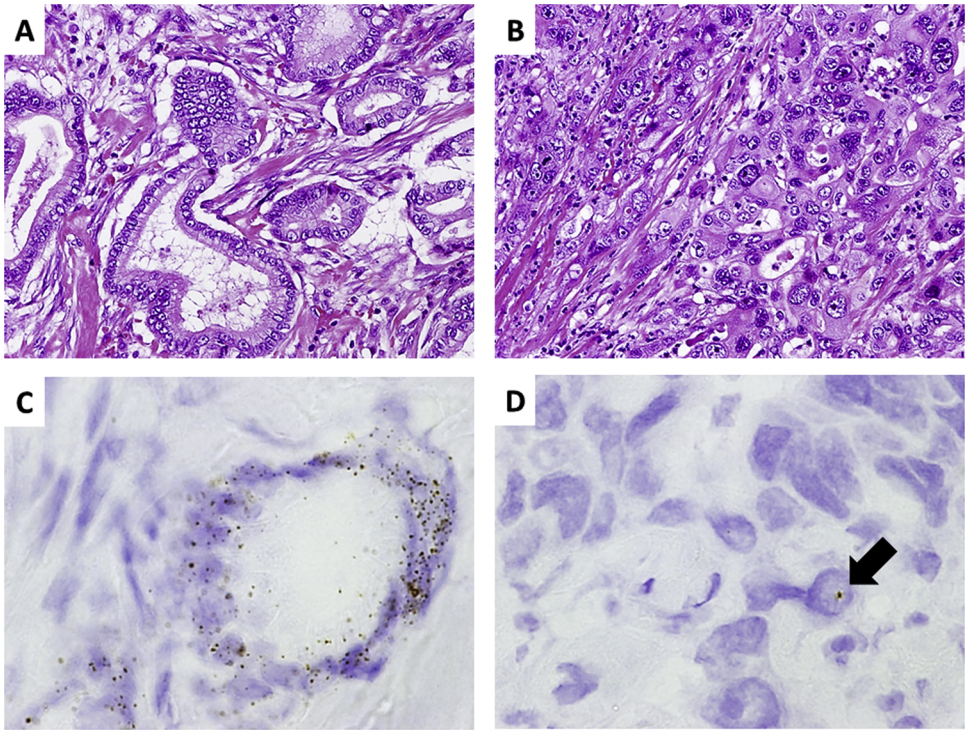

Fig. 2. *LGR5* expression in PDAC tissues. Representative HE staining images of low grade adenocarcinoma (A) and high grade adenocarcinoma (B). In low grade adenocarcinoma, abundant *LGR5* expression is in the gland-forming part (C). In high grade adenocarcinoma, *LGR5* expression was almost negative, but a small number of positive dots (arrow) is observed in only a few tumor cells (D). (A and B, HE, 200x; C and D, *LGR5* RNAscope, 400x).

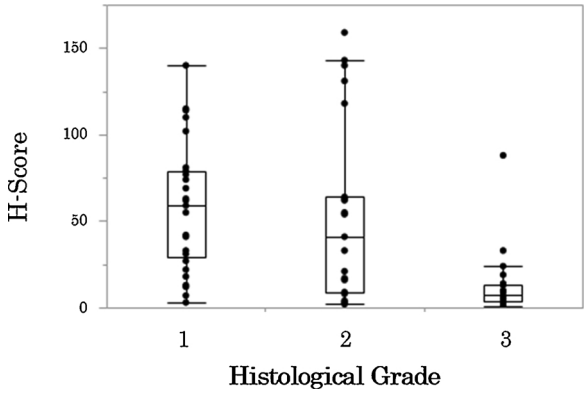

Fig. 3. Box-whisker plot of *LGR5* H-score during each histological grade. The H-score decreased as the histological grade increased.

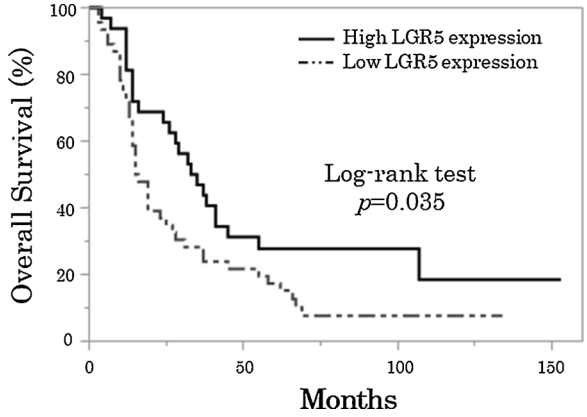

Fig. 4. Prognostic value of *LGR5* in PDAC by Kaplan–Meier analysis. The OS of patients with high *LGR5* expression was significantly longer than that of patients with low *LGR5* expression (median OS, 36 months (range, 4–153) vs. 17 months (range, 3–134), respectively; $p = 0.035$). The 5-year OS rates of patients with high and low *LGR5* expression were 21% and 17%, respectively.

3.4. Prognostic value of LGR5 in pancreatic ductal adenocarcinoma

We assessed the prognostic value of *LGR5* expression in PDAC patients using Kaplan–Meier analysis and log-rank test (Fig. 4). The median survival of the entire PDAC patient group was 26 months (range, 3–153) and the 5-year OS was 18%. The OS of the high *LGR5* expression patient group was significantly longer than the low *LGR5* expression patient group (median OS, 36 months (range, 4–153) vs. 17 months (range, 3–134), respectively; $p = 0.035$). The 5-year OS rates of the high and low *LGR5* expression groups were 21% and 17%, respectively.

We next evaluated the relationship of clinicopathological factors and *LGR5* expression with OS, using a Cox proportional hazard regression model (Table 2). In the univariate analysis, high histological grade ($p = 0.049$), lymph node involvement ($p < 0.001$), advanced clinical stage ($p < 0.001$) and low *LGR5* expression ($p = 0.034$) were significantly associated with worse OS. Variables that were statistically significant in the univariate analysis were entered into the multivariate analysis. Because lymph node status and clinical stage were strongly correlated, we adopted only the latter in the multivariate analysis. Multivariate analysis showed that clinical stage was an independent prognostic marker ($p = 0.009$), while *LGR5* expression did not remain a predictor of prognosis.

4. Discussion

In this study, *LGR5* expression was observed in all 78 PDAC patients with a wide range of expression, indicating that *LGR5* may be a CSC marker for PDAC. *LGR5* is overexpressed in various tumors, including colorectal cancer [12–14], gastric cancer [15–17], hepatocellular carcinoma [18] and esophageal cancer [19]. However, only a limited number of studies have evaluated *LGR5* expression in PDAC. In one report, Mizuno et al. examined *LGR5* expression by immunohistochemistry in nine PDAC patients who underwent resection; the authors found that *LGR5* was expressed in the cytoplasm in four of nine PDAC cases [22]. In another report, Simon et al. immunohistochemically investigated the prevalence and histoanatomical distribution of *LGR5* in tumors of the human gastrointestinal tract; *LGR5* expression was observed in all 17 PDAC tissues in their report [15]. This suggests that *LGR5* may be a CSC marker for PDAC, as in the other tumors described above. Several studies have identified markers for pancreatic CSCs, such as CD44, CD24, ESA, CD133 and c-MET [24–27]. Most of these markers have been demonstrated as CSC markers in other cancers. *LGR5* is the most promising and established stem cell marker and CSC marker in the gastrointestinal tract [28]. We thus considered that *LGR5* would be an excellent candidate for a CSC marker in pancreatic cancer.

Huch et al. reported that *Lgr5*-positive cells in the pancreas were capable of self-renewal and expansion *in vitro* [29]. Single isolated *LGR5*-positive cells could be cultured into pancreatic organoids, which showed that *Lgr5* positive cells represent stem cells for the pancreas. Furthermore, the authors found higher *Lgr5* expression in injured mice pancreas by partial duct ligation than in non-injured pancreas. Expression of *Lgr5* after injury has also been observed in the liver and stomach corpus as well as pancreas and its expression is presumed to be responsible for post-injury tissue regeneration [30,31]. In agreement with these findings, our results showed *LGR5* is expressed more highly in pancreatitis tissue compared with low expression in normal pancreas tissue.

Previous studies have evaluated the relationship between *LGR5* expression and clinical features in various kinds of cancers and found that *LGR5* expression is correlated with poor prognosis. The prognostic significance of *LGR5* expression was most extensively investigated in

Table 2
Univariate and multivariate analyses for prognosis factors of PDAC.

Factors	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Age: ≥ 65 years vs < 65 years	0.85 (0.52–1.43)	0.537		
Sex: male vs female	0.92 (0.57–1.50)	0.728		
Location of tumor: head vs body-tail	0.61 (0.35–1.01)	0.063		
Histological grade: low vs high	1.65 (1.00–2.77)	0.049	1.14 (0.61–2.17)	0.674
Tumor size: T1-2 vs T3-4	1.36 (0.71–2.43)	0.336		
Nodal metastasis: negative vs positive	2.89 (1.63–5.45)	< 0.001		
TNM stage: I-II vs III-IV	2.59 (1.52–4.33)	< 0.001	2.25 (1.23–4.12)	0.009
Adjuvant chemotherapy	0.60 (0.32–1.22)	0.153		
LGR5 expression: high vs low	1.71 (1.04–2.88)	0.034	1.17 (0.61–2.25)	0.639

colorectal cancer, and *LGR5* expression was correlated with worse clinical outcome [20,21]. However, some studies have reported conflicting results on the prognostic implications of *LGR5*, with no demonstrated link between *LGR5* expression and survival outcome in colorectal cancer [12,32]. One possible reason for the discrepancy in the prognostic implications may be the difference in the method to detect *LGR5* expression [17]. The studies that showed no link between *LGR5* expression and prognosis examined *LGR5* mRNA by RNA *in situ* hybridization or real time-PCR, whereas most of the studies that showed worse correlation of LGR5 expression with survival examined LGR5 protein by immunohistochemistry. Because the quality of immunohistochemical staining is insufficient to detect LGR5, this staining may represent a non-specific positive reaction and lead to inaccurate results [15]. Another study that examined LGR5 with another stem cell marker DCLK1 in gastric cancer by immunohistochemistry showed that both LGR5 and DCLK1 were highly expressed in well differentiated carcinoma and early stage carcinoma [33]. In our current study, we detected *LGR5* expression by RNAscope, which may be more sensitive than immunostaining, because it is a highly sensitive RNA *in situ* hybridization method.

The correlation between *LGR5* expression and clinical significance in PDAC patients has been unclear. In this study, *LGR5* expression was associated with better survival outcome in PDAC patients using the Kaplan–Meier method. Furthermore, *LGR5* expression was positively associated with low histological grade and early clinical stage. To the best of our knowledge, this is the first study showing the relationship between *LGR5* expression and clinicopathological characteristics and prognosis in PDAC. High histological grade tumors have a more aggressive biology, leading to earlier local and distant metastasis. In many studies, high histological grade of PDAC tumors was identified as a significantly worse prognostic factor than tumors with a lower tumor grade [34–36]. Furthermore, *LGR5* expression was associated with early clinical stage, which would naturally lead to the better prognosis. The better prognosis of PDAC patients with high *LGR5* expression may be associated with low histological grade and early clinical stage. Multivariate analysis revealed that LGR5 expression was not an independent prognostic marker. Bo Gun et al. examined *LGR5* expression in gastric cancer using RNAscope and found that *LGR5* positivity was high in tumors with low histological grade [17], which is in agreement with our findings. Walker et al. reported that loss of *Lgr5* expression may contribute to the invasive growth phenotype of colon carcinoma, while overexpression of *Lgr5* enhanced cell adhesion and attenuated invasiveness [37]. This study showed that PDAC patients with high *LGR5* expression are associated with low histological grade and early clinical stage, and these cases may be in the earlier stage of carcinogenesis. *LGR5* expression in PDAC appears to decline with tumor progression and dedifferentiation. CSCs are thought to play an important role in cancer formation. Our results suggest that *LGR5*-positive cells may function as CSCs in the initial phase of carcinogenesis rather than in tumor development and progression in PDAC. However, the exact mechanism by which *LGR5*-positive cells function in the development of PDAC remains unclear, and further examinations of *LGR5* expression in PDAC are required.

Our study has several limitations, including a small sample size that only included patients who underwent surgery at our hospital. Furthermore, we only examined RNA expression of *LGR5* in this study, and detection of CSC characteristics and *LGR5* expression analysis using pancreatic cancer cultured cells will be required in the future.

In conclusion, this study showed that *LGR5* may function as a CSC marker in PDAC patients and its higher expression was associated with low histological grade, early clinical stage and good prognosis in PDAC patients.

Author contribution

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

Declaration of Competing Interest

The authors state there are no conflicts of interest to disclose.

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