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Original Contribution

Correlation of Clinicopathological Features and *LGR5* Expression in Triple-Negative Breast Cancer

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ABSTRACT

LGR5 is the most robust known stem cell marker for gastrointestinal tumors, but there are few reports in breast cancer. Triple negative breast cancer (TNBC) is the most malignant subtype of breast cancer, and thus identification of new cancer stem cell populations in TNBC may help to identify targeted therapies. *LGR5* expression was evaluated by RNAscope, a newly developed RNA in situ hybridization technique, using a tissue microarray consisting of 43 patient samples of TNBC selected from the medical archives at our hospital. Patients were stratified into negative and positive *LGR5* expression groups. Tumor necrosis was greater in the *LGR5*-positive group compared with the *LGR5*-negative group ($P = .026$). Mitosis tended to show a high value in the *LGR5*-positive group compared with the *LGR5*-negative group ($P = .0831$), while stage tended to show a high stage in the *LGR5*-positive group compared with the *LGR5*-negative group ($P = .0617$). Cox proportional hazards models revealed that the *LGR5*-positive group (overall survival (OS) = 2.12; 95% CI: 2.12–2.12; $P = 0.1575$) had no relationship with OS. *LGR5* expression is associated with tumor necrosis of TNBC and suggested higher malignant potential.

1. Introduction

Breast cancer is the most common cancer in women and is the fifth leading cause of cancer death in woman [1]. Triple-negative breast cancer (TNBC), which shows an absence of ER, PgR, and HER2 expression, exhibits therapeutic resistance and has a poor prognosis [2]. It is reported that 10% to 20% of all breast cancers are TNBC [3].

In recent years, cancer stem cells have attracted attention as therapeutic targets, and breast cancer is no exception. In particular, *LGR5* is known to be a promising stem cell marker, but there are still few reports on breast cancer. It has also been reported as a cancer stem cell (CSC) marker in several other tumors including those of the colon [4-6], stomach [7-9], liver [10], and esophagus [11]. Several studies have analyzed *LGR5* expression and prognosis [12,13]. There are several reports that high *LGR5* expression is associated with poor prognosis, but this is controversial [14,15]. Therefore, here we report the analysis of *LGR5* expression and clinicopathological features using RNAscope.

2. Materials and methods

2.1. Patients

This study enrolled 43 patients with TNBC who underwent surgical resection between 2005 and 2013 at Shinshu University School of Medicine, Matsumoto, Japan. Clinicopathological data such as patient age and sex, pathological differentiation, and TNM classification were obtained by retrospective review of medical charts and pathological records. Prior to case selection, patients who received neoadjuvant chemotherapy were excluded. Special subtypes were also excluded. 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

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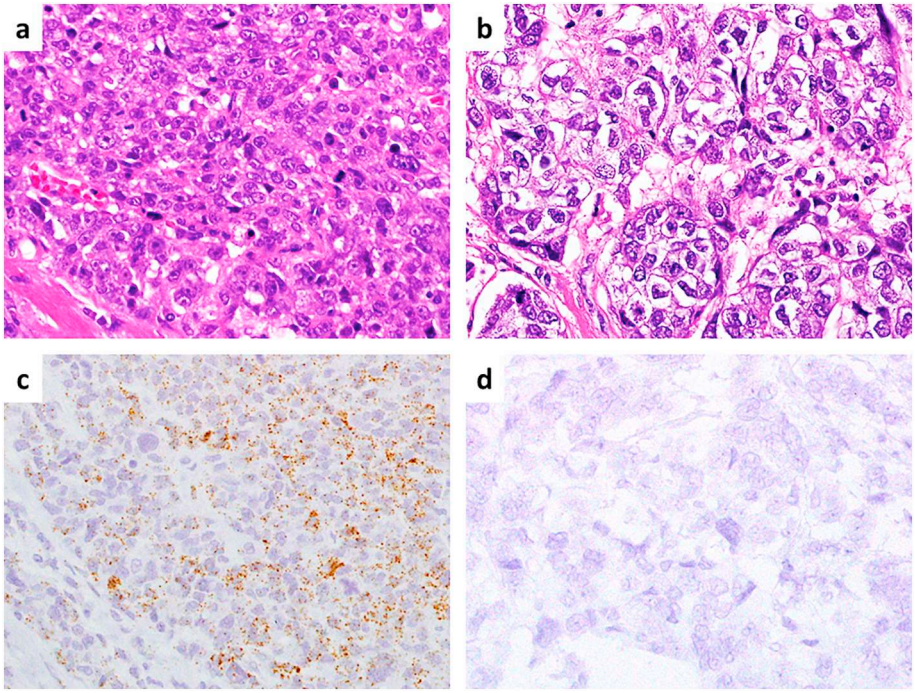

Fig. 1. Representative features of *LGR5* expression in TNBC. *LGR5*-positive (a and c) and -negative cases (b and d) (a and b, H&E; c and d, *LGR5* RNAscope).

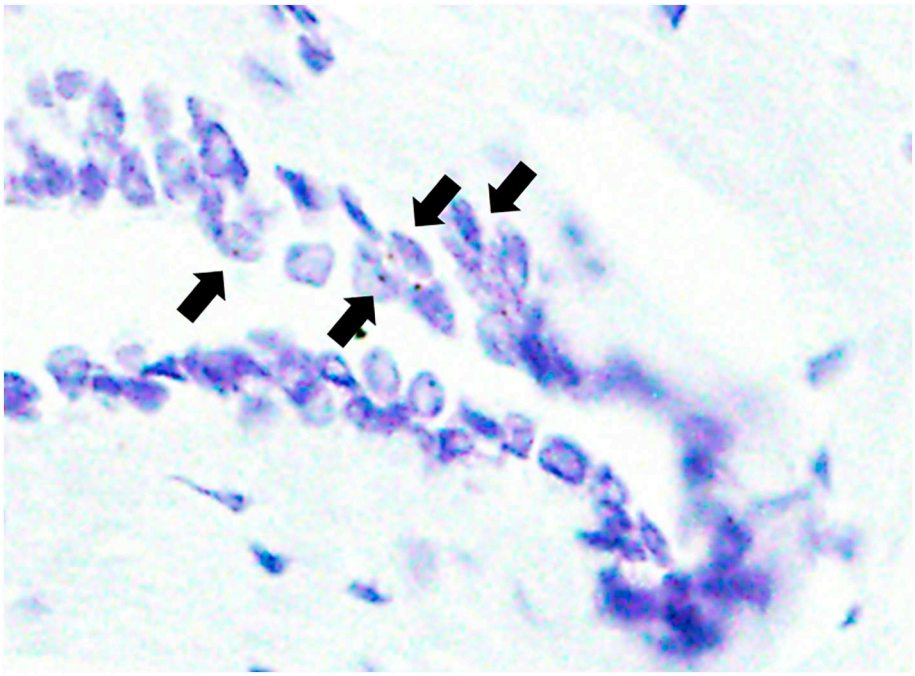

Fig. 2. *LGR5* expression in normal breast tissue. *LGR5*-positive dots (arrows) were observed in both glandular epithelial cells and myoepithelial cells in TDLU (*LGR5* RNAscope).

the Institutional Review Board of Shinshu University School of Medicine (approval No.4044).

2.2. Histopathology and immunohistochemistry

Formalin fixed paraffin embedded tissue were used for all specimens. Tissue microarray was made with tissue containing tumor. Each tumor block was punched out with steel needles to a diameter of 3 mm and the punched cores are arranged in new blocks. 4-μm thick serial sections were made from these blocks and stained for H&E. We compared the nuclear grade [16]: grade 1 and 2 were classified as low and 3 as high. Immunohistochemistry with β-catenin (1:500: Becton-Dickinson & Company, Franklin Lakes, NJ, USA) and Ki67 (1:100: Dako, Glostrup, Denmark) antibodies using the EnVision method. The percentage of cells staining positive for Ki67 was calculated from at least three different areas per case. Ki67 expression was grouped as $<33\%$, 34% to 66%, and 67% to 100%. $>33\%$ of cases were classified as high Ki67. A case was considered β-catenin-positive if 10% or more of the tumor cells expressed nuclear-translocated β-catenin.

Table 1
LGR5 expression and clinicopathological characteristics in TNBC.

Factors	n	*LGR5* expression		*P*-value
		Positive (n = 7)	Negative (n = 36)	
Age				0.3633
64 years	19	2	17	
<64 years	24	5	19	
LN metastasis				0.7355
Present	16	3	13	
Absent	27	4	23	
Nuclear grade				0.2114
High	28	6	22	
Low	15	1	14	
Necrosis				0.026
Present	27	7	20	
Absent	16	0	16	
Ki67 score				0.0831
High	18	5	13	
Low	25	2	23	
TNM stage				0.0617
I	20	1	19	
II–IV	23	6	17	

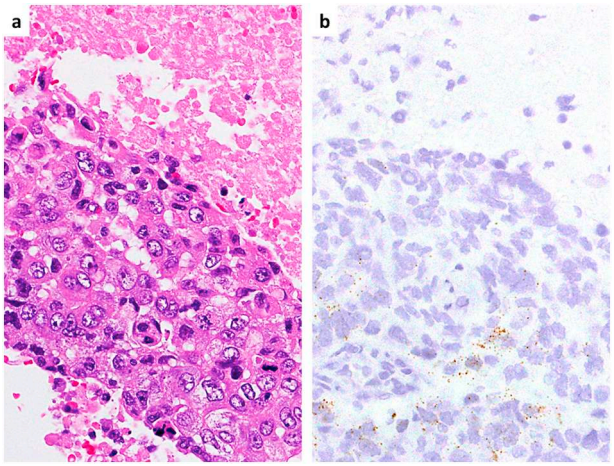

Fig. 3. Representative features of TNBC with necrosis. High levels of *LGR5* expression were observed with necrosis (a and b) (a, H&E; b, *LGR5* RNAscope).

Table 2
Univariate analyses of prognostic factors for TNBC.

Factors	Univariate analysis	
	HR (95% CI)	*P*-value
Age: ≧64 years vs <64years	2.38 (0.39–18.33)	0.3401
Ki67 score: low vs. high	0.36 (0.02–2.46)	0.3231
Nuclear grade: low vs high	0.31 (0.04–1.89)	0.1978
Necrosis	0.14 (0.01–0.98)	0.0469
TNM stage: I vs. II–IV	3.01 (0.44–59.40)	0.2773
LGR5 expression: negative vs. positive	2.12 (2.12–2.12)	0.1575

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 by heating and protease application prior to hybridization with a *LGR5*-specific probe. The detailed procedure is described in an earlier publication [17]. 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 on the basis of the H-score per high-power field (400× magnification). Furthermore, H-score of 10 or more was regarded as *LGR5*-positive. We analyzed the relationship between *LGR5* expression and clinicopathological data and prognosis in TNBC 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 test was adopted to test for differences between subgroups of patients. The OS rates of TNBC patients were calculated using the Kaplan-Meier method, and differences in those rates were compared using 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).

3. Results

3.1. LGR5 expression in breast carcinoma and normal breast tissue

A wide range of expression levels was observed from cases in which a few dots were found in a few cells to those in which a large number of dots were found in a large number of cells (Fig. 1a, c). In many cases, *LGR5* expression could not be detected at all (Fig. 1b, d). On the other hand, there was no particular tendency in the distribution of expressing cells. Seven cases could be recognized as the *LGR5* high expression group.

Epithelial cells with *LGR5*-positive dots in the terminal duct lobular units (TDLU) were identified, and *LGR5*-positive cells were observed in both glandular epithelial cells and myoepithelial cells (Fig. 2). *LGR5* expression was also identified in epithelium of larger ducts other than TDLU.

3.2. LGR5 expression and clinicopathological characteristics in breast carcinoma

The clinicopathological characteristics of the TNBC patients are listed in Table 1. Necrotic foci were significantly frequent in the *LGR5* high expression group (Fig. 3a, b). When stage was divided into I and II–IV, stages II–IV were more frequent in the high *LGR5* expression group. In the *LGR5* high expression group, Ki67 positivity was also more frequent. The expression of nuclear-translocated β-catenin was not identified in all cases.

3.3. Prognostic value of LGR5 in breast carcinoma

The prognostic value of *LGR5* expression in TNBC was analyzed by Kaplan-Meier analysis with the log-rank test. The median OS rate for the study patients was 2743 days (range, 2050–3640). There was no significant difference in OS between TNBC cases in the *LGR5*-positive group (median OS, 2743 days (range, 1960–3948)) and the *LGR5*-negative group (median OS, 2753 days (range, 2289–3410)) (log rank test, $P = 0.2929$).

We evaluated the relationship between clinicopathological factors and *LGR5* expression with OS using a Cox proportional hazard regression model (Table 2). There was no correlation with *LGR5* (HR = 2.12; 95% CI: 2.12–2.12; $P = 0.1575$).

4. Discussion

To our knowledge, this is the first report of RNA in situ expression analysis of *LGR5* in breast cancer. The expression levels of *LGR5* in TNBC breast cancer were found to vary and there were negative cases. Multipotent mammary stem cells that can differentiate into both myoepithelial cells and luminal lineage cells, and unipotent mammary stem cells that differentiate only into either myoepithelial cells or luminal lineage cells have been reported as mammary stem cells [18]. *LGR5* expression was reported in both multipotent mammary stem cells and unipotent mammary stem cells. Similarly, in our study, *LGR5* expression was identified in both glandular epithelial cells and myoepithelial cells in TDLU. Therefore, it may be possible that *LGR5* is a CSC marker for TNBC.

However, *LGR5* is not normally expressed in normal gastric fundic glands, but recently it has been reported that it is expressed in gastric fundic glands under specific conditions [19]. Although *LGR5* was negative in many cases of TNBC, *LGR5* expression ability is not lacking in TNBC, and *LGR5* may only be expressed under special conditions. Because *LGR5*-positive TNBC is significantly more likely to be associated with necrosis and mitosis tends to be higher, *LGR5* expression may be influenced by such special conditions.

High expression of CD44, another breast CSC marker, in TNBC was associated with necrosis and high Ki67 was linked to high mitosis [20]. These reports may enhance the possibility that *LGR5* is a CSC marker in TNBC.

Although there is no report on the association between *LGR5* and prognosis in TNBC, relapse-free survival has been reported to deteriorate in non-TNBC [21]. Therefore, the relationship with disease stage in our study may have an influence on prognosis although there is no difference in OS. Nonetheless, although the association with prognosis was controversial in colon cancer and other cancer types, examination with the RNAscope method has been reported as having a good prognosis in recent years [22]. In colon cancer, it is known that nuclear translocation of β-catenin resulting from an abnormality of APC signaling affects *LGR5* expression. However, no β-catenin nuclear translocation was observed in this study, and *LGR5* expression resulting from nuclear translocation of β-catenin does not seem to be related, at least in TNBC.

In TNBC, *LGR5* was not expressed in all cases, and the appearance of other promising markers in terms of CSCs as a therapeutic target in TNBC is expected. However, it also became clear that an *LGR5*-positive group existed. *LGR5*-expressing tumors may be a subtype of TNBC. These clinicopathological characterizations require further study.

A limitation of this study is that we did not analyze *LGR5* expression in all cancer tissue areas, and its expression may vary and be associated with other clinicopathological characteristics. Furthermore, *LGR5* expression analysis by other experimental approaches will also be required.

In conclusion, we demonstrated *LGR5* expression in TNBC by RNA in situ. Further research in the future may reveal *LGR5* as a promising therapeutic target.

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Author contribution

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

Declaration of competing interest

The authors declare no conflict of interest.

References

[1] Global Burden of Disease Cancer C, Fitzmaurice C, Akinyemiju TF, et al. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-Years for 29 Cancer Groups, 1990 to 2016: A Systematic Analysis for the Global Burden of Disease Study. JAMA Oncol 2018; 4:1553–1568.
[2] Dent R, Trudeau M, Pritchard KI, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 2007;13:4429–34.
[3] Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med 2010;363:1938–48.
[4] Ziskin JL, Dunlap D, Yaylaoglu M, et al. In situ validation of an intestinal stem cell signature in colorectal cancer. Gut 2013;62:1012–23.
[5] He S, Zhou H, Zhu X, et al. Expression of Lgr5, a marker of intestinal stem cells, in colorectal cancer and its clinicopathological significance. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 2014; 68:507–513.
[6] Uchida H, Yamazaki K, Fukuma M, et al. Overexpression of leucine-rich repeat-containing G protein-coupled receptor 5 in colorectal cancer. Cancer Sci 2010;101:1731–7.
[7] Simon E, Petke D, Boger C, et al. The spatial distribution of LGR5+ cells correlates with gastric cancer progression. PLoS One 2012;7:e35486.
[8] Yamanoi K, Fukuma M, Uchida H, et al. Overexpression of leucine-rich repeat-containing G protein-coupled receptor 5 in gastric cancer. Pathol Int 2013;63:13–9.
[9] Jang BG, Lee BL, Kim WH. Prognostic significance of leucine-rich-repeat-containing G-protein-coupled receptor 5, an intestinal stem cell marker, in gastric carcinomas. Gastric Cancer 2016;19:767–77.
[10] Fukuma M, Tanese K, Effendi K, et al. Leucine-rich repeat-containing G protein-coupled receptor 5 regulates epithelial cell phenotype and survival of hepatocellular carcinoma cells. Exp Cell Res 2012;319:113–21.
[11] Becker L, Huang Q, Mashimo H. Lgr5, an intestinal stem cell marker, is abnormally expressed in Barrett's esophagus and esophageal adenocarcinoma. Dis Esophagus 2010;23:168–74.
[12] Jiang Y, Li W, He X, et al. Lgr5 expression is a valuable prognostic factor for colorectal cancer: evidence from a meta-analysis. BMC Cancer 2016;16:12.
[13] Han Y, Xue X, Jiang M, et al. LGR5, a relevant marker of cancer stem cells, indicates a poor prognosis in colorectal cancer patients: a meta-analysis. Clin Res Hepatol Gastroenterol 2015;39:267–73.
[14] Takahashi H, Ishii H, Nishida N, et al. Significance of Lgr5(+ve) cancer stem cells in the colon and rectum. Ann Surg Oncol 2011;18:1166–74.
[15] Martin ML, Zeng Z, Adileh M, et al. Logarithmic expansion of LGR5(+) cells in human colorectal cancer. Cell Signal 2018;42:97–105.
[16] Tsuda H, Akiyama F, Kurosumi M, et al. The efficacy and limitations of repeated slide conferences for improving interobserver agreement when judging nuclear atypia of breast cancer. The Japan National Surgical Adjuvant Study of Breast Cancer (NSAS-BC) Pathology Section. Jpn J Clin Oncol 1999;29:68–73.
[17] Ukpo OC, Flanagan JJ, Ma XJ, et al. High-risk human papillomavirus E6/E7 mRNA detection by a novel in situ hybridization assay strongly correlates with p16 expression and patient outcomes in oropharyngeal squamous cell carcinoma. Am J Surg Pathol 2011;35:1343–50.
[18] Zhou J, Chen Q, Zou Y, et al. Stem cells and cellular origins of mammary gland: updates in rationale, controversies, and cancer relevance. Stem Cells Int 2019;2019:4247168.
[19] Leushacke M, Tan SH, Wong A, et al. Lgr5-expressing chief cells drive epithelial regeneration and cancer in the oxyntic stomach. Nat Cell Biol 2017;19:774–86.
[20] Liu M, Tsang JYS, Lee M, et al. CD147 expression is associated with poor overall survival in chemotherapy treated triple-negative breast cancer. J Clin Pathol 2018;71:1007–14.
[21] Hou MF, Chen PM. Chu PY LGR5 overexpression confers poor relapse-free survival in breast cancer patients. BMC Cancer 2018;18:219.
[22] Jang BG, Kim HS, Chang WY, et al. Expression profile of LGR5 and its prognostic significance in colorectal cancer progression. Am J Pathol 2018;188:2236–50.