Characterization of LGR5 expression in poorly differentiated colorectal carcinoma with mismatch repair protein deficiency

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Abstract

Background Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is a promising intestinal stem cell and carcinoma stem cell marker. We examined the relationship between mismatch repair (MMR) protein deficiency and LGR5 expression in poorly differentiated (PD) colorectal carcinoma (CRC). Methods In 29 cases of PD-CRC, deficiencies in MMR proteins (MLH1, PMS2, MSH2, MSH6) and β-catenin expression were identified by immunohistochemistry (IHC). LGR5 expression was examined by RNAscope assay in tissue microarrays. Results LGR5 H-scores in MMR-deficient (MMR-D) cases were significantly lower than those in MMR-proficient (MMR-P) cases (P =0.0033). Nuclear β-catenin IHC scores in MMR-D cases were significantly lower than those in MMR-P cases (P =0.0024). In all cases, there was a positive correlation between LGR5 H-score and nuclear β-catenin IHC score (r=0.6796, P <0.001). Even in MMR-D and MMR-P cases, there was positive correlation between LGR5 H-score and nuclear β-catenin IHC score (r=0.7180, P <0.0085 and r=0.6574, P <0.003, respectively). MMR-D CRC cases showed low expression of LGR5 , which might be due to low activation of the Wnt/β-catenin signaling pathway. Conclusion Our results reveal the relationship between LGR5 expression and MMR protein profiles in PD-CRC. A further study is warranted to confirm these findings.

Background

Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is a promising intestinal stem cell marker [1]. LGR5 is also a candidate marker for colorectal cancer (CRC) stem cells [2] and may be closely involved in the progression and prognosis of CRC [3]. However, Jang et al. suggested a suppressive role for LGR5 in CRC progression [4].

CRC is one of the most common carcinomas worldwide [5] and is classified into well, moderately, poorly, and undifferentiated types according to gland structures. The colon carcinogenesis model is roughly divided into an adenoma–carcinoma sequence and microsatellite instability (MSI) [6]. Approximately 90% of CRCs have APC inactivation, which has a major role in adenoma formation, and subsequent multistage mutations such as KRAS and TP53 cause carcinogenesis [7, 8]. MSI is a hypermutable phenotype caused by abnormalities in DNA repair. Mismatch repair (MMR) proteins such as MLH1, MSH2, MSH6, and PMS2 are inactivated, and gene mutations accumulate. Lynch syndrome suffers from germline mutations in MMR-related genes, which induces tumors such as CRC [9]. Methylation in the promoter regions of MMR genes promotes suppression of MMR protein expression, defining a carcinogenesis pathway of CRC that differs from the classic adenoma–carcinoma sequence [10].

Poorly differentiated (PD)-CRC has a poor prognosis compared with well and moderately differentiated CRC [11]. However, PD-CRC with MSI has a low lymph node metastasis rate and shows a good prognosis [12] but the mechanisms that define its clinicopathological differences have not yet been clarified. In this study, a new analysis focusing on LGR5-positive carcinoma stem cells was performed to investigate differences in prognosis based on a carcinogenic model of PD-CRC. We also investigated the relationship

between LGR5 and β-catenin expression related to LGR5 regulation, and that between CD8-positive tumor-infiltrating lymphocytes (CD8 + TIL) and LGR5 expression in immune responses.

Methods

Patients and materials

We identified 29 cases of PD-CRC at Shinshu University Hospital, Matsumoto, Japan from 2004 to 2014, and evaluated their clinicopathological features.

Histopathology, immunohistochemical staining, and evaluation

All samples were fixed in 20% formaldehyde and paraffin tumor blocks were made. Tumor blocks of CRC were selected to prepare a tissue microarray (TMA). The most representative region of each CRC sample was selected. Tissue cores were punched out from each 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 hematoxylin and eosin (HE), and immunostained with MutL protein homolog 1 (MLH1) (ES05, mouse monoclonal, dilution 1:50), postmeiotic segregation increased 2 (PMS2) (EP51, rabbit monoclonal, dilution 1:40), MutS protein homolog 2 (MSH2) (FE11, mouse monoclonal, dilution 1:50), MutS protein homolog 6 (MSH6) (EP49, rabbit monoclonal, dilution 1:50; Agilent Technologies, Santa Clara, CA), β-catenin (mouse monoclonal, dilution 1:500; Becton-Dickinson & Company, Franklin Lakes, NJ, USA), or CD8 (CD8/144B, mouse monoclonal, dilution 1:50; Dako, Copenhagen, Denmark) antibodies. For antigen retrieval methods, sections were boiled in 0.05% citraconic anhydride solution, pH7.4 (Immunosaver; Nissin EM, Tokyo, Japan) for 45 min for MLH1, PMS2, MSH2, and MSH6, or microwaved in 0.45%Tris-5mM EDTA for 25min for β-catenin and CD8. the detection of MMR proteins using the NovoLink polymer detection system (Leica Microsystems GmbH, Wetzlar, Germany) and β-catenin and CD8 using the Envision detection system (Agilent Technologies, Santa Clara, CA) according to the manufacturers' recommendations.

The immunohistochemical intensity of MLH1, PMS2, MSH2, and MSH6 were classified into four grades from 0 to 3. We defined grade 0–1 as downregulated expression and grade 2–3 as stable expression. We determined cases of PD-CRC as having a MMR protein deficiency when at least one of MLH1, PMS2, MSH2, and MSH6 was grade 0–1.

β-catenin staining was evaluated as previously described [13]. The results were calculated as IHC scores, where IHC score = percentage of nuclear positive cells × staining intensity. Nuclear staining was classified into five grades from 0 to 4. We defined staining intensity as follows: 0; negative, 1; weak, 2; moderate, 3; strong, and 4; very strong. The nuclear β-catenin IHC score ranged from 0 to 400. The number of CD8+ TILs was calculated in the three most infiltrated fields in each case.

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 was applied prior to hybridization with a *LGR5*-specific probe. The detailed procedure is described in a previous publication [14]. Brown dots present in the nucleus and/or cytoplasm were recognized as positive staining. *LGR5* expression was quantified using a five-level 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 one or more dots was regarded as *LGR5*-positive.

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 correlations. The Wilcoxon rank sum test or chi-squared test was also applied to assess the statistical significance. A P value of < 0.05 was considered significant.

Results

Correlation between clinicopathological factors and MMR protein expression

We first evaluated the expression of MMR proteins (MLH1, PMS2, MSH2, and MSH6) in 29 PD-CRC cases. Among the cases, 17 cases were MMR protein-proficient (MMR-P) and 12 cases were MMR protein-deficient (MMR-D) adenocarcinoma. Representative images and staining of MMR-D and MMR-P cases are shown in Fig. 1. Table 1 summarizes the correlation between clinicopathological factors and MMR protein expression. MMR-D cases were significantly correlated with age, tumor site, lymph node metastasis, pathological stage, and CD8+ TILs (Figs. 2A, 2B, 2D and 2E).

LGR5 RNA expression in PD-CRC with MMR protein deficiency

We evaluated *LGR5* expression in all cases. All 29 cases contained carcinoma cells with some *LGR5*-positive dots, with a wide range of *LGR5*-positive cell staining. Representative images of *LGR5* staining in MMR-D and MMR-P are shown in Figure 2C and 2F. *LGR5* H-scores varied among the cases. Mean H-scores for *LGR5* staining in MMR-P and MMR-D cases were 62.9 (24.2–136.2) and 24.4 (7.9–63.4), respectively. *LGR5* H-scores in MMR-D cases were significantly lower than in MMR-P cases (P=0.034) (Fig.3).

Correlation between *LGR5* expression and β-catenin expression

Previous studies in CRC showed that β-catenin is related to *LGR5*, which is a cancer stem cell (CSC) marker [15], and that β-catenin induces the expression of *LGR5* [16]. We thus analyzed the correlation between *LGR5* H-score and the expression of nuclear-translocated β-catenin. Mean nuclear β-catenin IHC scores in MMR-P cases and MMR-D cases were 104.5 (81.3–285.8) and 23.9 (9.9–77.1), respectively. Nuclear β-catenin IHC scores in MMR-D cases were significantly lower than in MMR-P cases (P=0.002). In all cases, there was a positive correlation between *LGR5* H-score and nuclear β-catenin IHC score (r=0.728, P<0.001). Even in MMR-D and MMR-P cases, there was a positive correlation between *LGR5* H-score and nuclear β-catenin IHC score (r=0.692, P<0.013 and r=0.679, P=0.003, respectively)

Discussion

Most CRC cases show multistep carcinogenesis based on an adenoma–carcinoma sequence [8, 17]. In the first step, inactivation of APC induces aberrant Wnt/β-catenin activation and contributes to tumorigenesis [18]. LGR5 is the target gene of β-catenin and TCF/LEF complex, and nuclear-translocated β-catenin affects LGR5 expression, as shown by previous reports and our current data [16, 19]. Deficiencies in MMR proteins are caused by genetic or epigenetic alterations in MLH1, PMS2, MSH2, and MSH6. Deficiencies in MMR proteins induce microsatellite instability and lead to the acquisition of various gene mutations [20]. In MSI carcinoma, genetic mutations such as BRAF have been reported, while driver mutations frequently observed in CRC are infrequent [21]. In this study, morphologically similar CRC was classified into two biologically different groups based on the expression of MMR proteins, and their LGR5 expression and clinicopathological features were analyzed.

Although the expression levels of LGR5 differ between MMR-D and MMR-P, each correlation with β-catenin proves the association between β-catenin and LGR5 expression. Low expression of LGR5 in MMR-D may be related to low β-catenin expression. Furthermore, these may be linked to abnormalities related to Wnt/β-catenin signaling such as APC abnormalities.

There is controversy regarding the relationship between LGR5 expression and prognosis in CRC. One issue is that MMR-D and MMR-P were pooled and analyzed together in previous studies. Another issue is the LGR5 evaluation method. High expression of LGR5 has been reported to be associated with poor prognosis, but this is controversial. It may be because many prognostic evaluations are performed by immunohistostaining. In a report using the RNA in situ hybridization method, high expression of LGR5 was observed in well differentiated CRC and showed better prognosis [4]. Therefore, the high frequency of the MMR-D group with PD-CRC may be due to the low expression of LGR5 in this group.

A recent report described differences in the presence of LGR5-positive carcinoma stem cells based on the differences in MMR protein expression [4]. However, no study has previously analyzed LGR5 expression in detail in cases of PD-CRC alone. In this study, we demonstrated LGR5 expression was significantly lower

in MMR-D. The deficiency in MMR proteins contributed to an increase in tumor mutation burden in tumor tissue. A characteristic of MSI carcinoma is the recruitment of infiltrating inflammatory cells due to increased antigen presentation by anti-cancer host immunity. Previous reports and our data also suggest CD8 + TILs were high in frequency in MMR-D CRC [22]. The good prognosis of PD-CRC with MMR-D despite the low expression of LGR5 may be due to differences in immune responses. Assessment of LGR5 as a prognostic marker may enable CRC to be divided into MMR-D and MMR-P and should be assessed by RNA in situ hybridization. Analysis of a larger number of PD-CRC with MMR-D cases is warranted for more robust data. Although LGR5 is a stem cell marker for CRC, classification into several subgroups may lead to LGR5 being a more sensitive prognostic marker.

There are some limitations in our study. First, we did not examine mutations related to Wnt/β-catenin signaling such as APC, so these may need to be compared with LGR5. Furthermore, it is necessary to investigate the relationship between APC abnormalities and LGR5 using cultured cells.

Conclusion

Our results reveal the relationship between LGR5 expression and MMR protein profiles in PD-CRC. A further study is warranted to confirm these findings.

Abbreviations

LGR5: Leucine-rich repeat-containing G-protein-coupled receptor 5; MMR: mismatch repair; PD: poorly differentiated; CRC: colorectal carcinoma; IHC: immunohistochemistry; MMR-D: MMR-deficient; MMR-P: MMR-proficient; colorectal carcinoma: CRC; MSI: microsatellite instability; TIL: tumor-infiltrating lymphocytes; TMA: tissue microarray; MLH1: MutL protein homolog 1; PMS2: postmeiotic segregation increased 2; MSH2: MutS protein homolog 2; MSH6: MutS protein homolog 6

Declarations

Author contribution

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

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Availability of data and materials

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

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.

Competing interests

The authors declare that they have no competing interests.

References

1. Barker N, Clevers H Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells. Gastroenterology. 2010;138:1681-1696.
2. de Sousa e Melo F, Kurtova AV, Harnoss JM, Kljavin N, Hoeck JD, Hung Jet al. A distinct role for Lgr5(+) stem cells in primary and metastatic colon cancer. Nature. 2017;543:676-680.
3. Wu XS, Xi HQ, Chen L Lgr5 is a potential marker of colorectal carcinoma stem cells that correlates with patient survival. World J Surg Oncol. 2012;10:244.
4. Jang BG, Kim HS, Chang WY, Bae JM, Kim WH, Kang GH Expression Profile of LGR5 and Its Prognostic Significance in Colorectal Cancer Progression. Am J Pathol. 2018;188:2236-2250.
5. Navarro M, Nicolas A, Ferrandez A, Lanas A Colorectal cancer population screening programs worldwide in 2016: An update. World J Gastroenterol. 2017;23:3632-3642.
6. Fearon ER, Vogelstein B A genetic model for colorectal tumorigenesis. Cell. 1990;61:759-767; Thibodeau SN, Bren G, Schaid D Microsatellite instability in cancer of the proximal colon. Science. 1993;260:816-819.
7. Ando M, Takemura K, Maruyama M, Endo M, Iwama T, Yuasa Y Mutations in c-K-ras 2 gene codon 12 during colorectal tumorigenesis in familial adenomatous polyposis. Gastroenterology. 1992;103:1725-1731; Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JMet al. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science. 1989;244:217-221; Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B Suppression of human colorectal carcinoma cell growth by wild-type p53. Science. 1990;249:912-915; Ichii S, Horii A, Nakatsuru S, Furuyama J, Utsunomiya J, Nakamura Y Inactivation of both APC alleles in an early stage of colon adenomas in a patient with familial adenomatous polyposis (FAP). Hum Mol Genet. 1992;1:387-390.

8. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert Met al. Genetic alterations during colorectal-tumor development. The New England journal of medicine. 1988;319:525-532.
9. Boland CR, Goel A Microsatellite instability in colorectal cancer. Gastroenterology. 2010;138:2073-2087 e2073.
10. Martin SA, Lord CJ, Ashworth A Therapeutic targeting of the DNA mismatch repair pathway. Clin Cancer Res. 2010;16:5107-5113.
11. Halvorsen TB, Seim E Degree of differentiation in colorectal adenocarcinomas: a multivariate analysis of the influence on survival. J Clin Pathol. 1988;41:532-537.
12. Lagerstedt Robinson K, Liu T, Vandrovcova J, Halvarsson B, Clendenning M, Frebourg Tet al. Lynch syndrome (hereditary nonpolyposis colorectal cancer) diagnostics. J Natl Cancer Inst. 2007;99:291-299.
13. Wong SC, Lo ES, Chan AK, Lee KC, Hsiao WL Nuclear beta catenin as a potential prognostic and diagnostic marker in patients with colorectal cancer from Hong Kong. Mol Pathol. 2003;56:347-352; Wong SC, Lo SF, Lee KC, Yam JW, Chan JK, Wendy Hsiao WL Expression of frizzled-related protein and Wnt-signalling molecules in invasive human breast tumours. J Pathol. 2002;196:145-153.
14. Ukpo OC, Flanagan JJ, Ma XJ, Luo Y, Thorstad WL, Lewis JS, Jr. 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;35:1343-1350.
15. Fan XS, Wu HY, Yu HP, Zhou Q, Zhang YF, Huang Q Expression of Lgr5 in human colorectal carcinogenesis and its potential correlation with beta-catenin. Int J Colorectal Dis. 2010;25:583-590.
16. Clevers H, Nusse R Wnt/beta-catenin signaling and disease. Cell. 2012;149:1192-1205.
17. Leslie A, Carey FA, Pratt NR, Steele RJ The colorectal adenoma-carcinoma sequence. Br J Surg. 2002;89:845-860.
18. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Jr., Kinzler KW Cancer genome landscapes. Science. 2013;339:1546-1558.
19. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone Aet al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell. 2002;111:241-250; Van der Flier LG, Sabates-Bellver J, Oving I, Haegebarth A, De Palo M, Anti Met al. The Intestinal Wnt/TCF Signature. Gastroenterology. 2007;132:628-632.
20. Smyrk TC, Watson P, Kaul K, Lynch HT Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma. Cancer. 2001;91:2417-2422.
21. Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda Met al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut. 2009;58:90-96; Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MAet al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet. 2006;38:787-793.

22. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring ADet al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. The New England journal of medicine. 2015;372:2509-2520.

Table

Table 1 Correlation between clinicopathological factors and MMR protein expression

		MMR protein		
	n	Proficient	Deficient	*P*-value
Sex				
Male	14	7	7	0.363
Female	15	10	5	
Age				
≥71	15	12	3	0.016*
<71	14	5	9	
Tumor site				
Proximal	17	7	10	0.023*
Distal	12	10	2	
Lymphovascular invasion				
Absent	0	0	0	NA
Present	29	17	12	
Lymph node metastasis				
Absent	9	0	9	<0.001*
Present	20	17	3	
Pathological stage				
≤pT2	7	0	7	<0.001*
>pT3	22	17	5	
CD8+ TILs				
≤155.7	15	15	0	<0.001*
>155.7	14	2	10	

TIL, tumor-infiltrating lymphocytes.

Figures

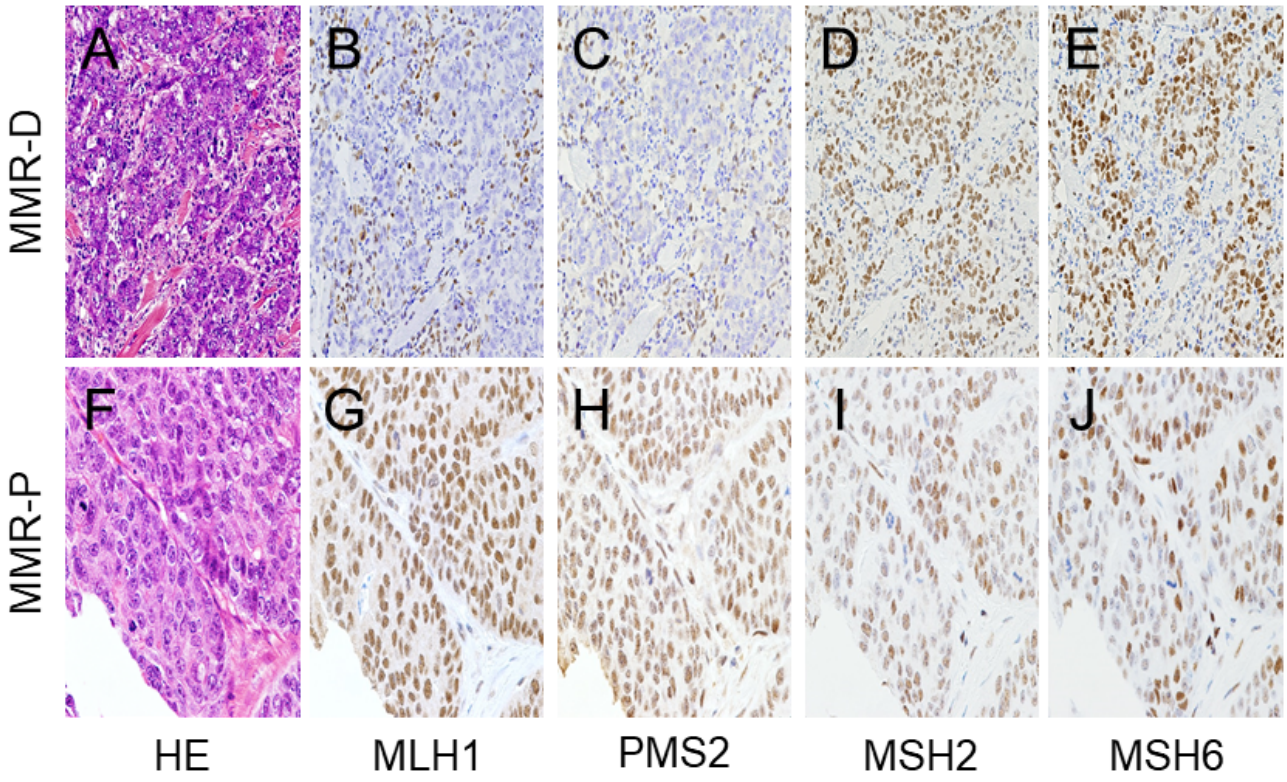

Figure 1

Representative images and staining of MMR-P and MMR-D. Representative features in MMR-D (A) and MMR-P (F). Immunohistochemistry of four MMR proteins. Loss of MLH1/PMS2 (B and C) and expression of MSH2/MSH6 (D and D) are shown in MMR-D. All four MMR proteins (G, H, I, and J) are shown in MMR-P. (A and F, HE; B and G, MLH1 immunostaining; C and H, PMS2 immunostaining; D and I, MSH2 immunostaining; E and J, MSH6 immunostaining.)

Figure 2

Representative images of LGR5 and CD8 in MMR-P and MMR-D. Representative features in MMR-D (A) and MMR-P (D). In MMR-D, many CD8 positive lymphocytes were observed (B) and LGR5 expression was low (C). In MMR-P, few CD8-positive lymphocytes were observed (E) and high levels of LGR5 expression was observed (F). (A and D, HE; B and E, CD8 immunostaining; C and F, LGR5 RNAscope.)

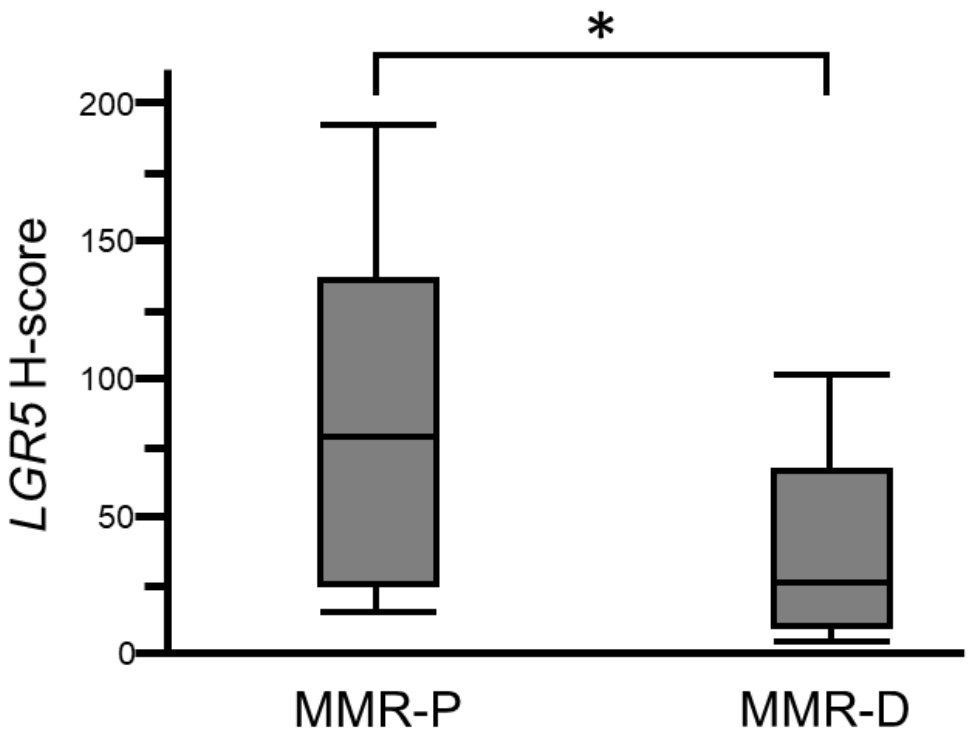

Figure 3

Box plot of LGR5 H-scores in MMR-P and MMR-D. LGR5 H-scores in MMR-D cases were significantly lower than those in MMR-P cases (P=0.034).