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Overexpression of leucine-rich repeat-containing G protein-coupled receptor 5 in colorectal cancer

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Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) is a 7-transmembrane receptor reportedly expressed in stem cells of the intestinal crypts and hair follicles of mice. Overexpression of LGR5 is observed in some types of cancer; however, there has been no specific assessment in colorectal tumorigenesis. We performed quantitative RT-PCR for LGR5 expression in 37 representative cancer cell lines, and showed that LGR5 mRNA was frequently overexpressed in colon cancer cell lines. Moreover, LGR5 expression was higher in colon cancer cell lines derived from metastatic tumors compared with those from primary tumors. In clinical specimens, there was significant overexpression of LGR5 in 35 of 50 colorectal cancers (CRCs), and in seven of seven sporadic colonic adenomas, compared with matched normal mucosa. This suggests up-regulation of LGR5 from the early stage of colorectal tumorigenesis. LGR5 expression showed marked variation among CRC cases and correlated significantly with lymphatic invasion, vascular invasion, tumor depth, lymph node metastasis, and tumor stage (IIIC *vs.* IIIB). In addition to cancer cells, crypt base columnar cells of the small intestine and colon were shown by *in situ* hybridization to express LGR5. This is the first report suggesting the involvement of LGR5, not only in early events but also in late events in colorectal tumorigenesis. (*Cancer Sci* 2010; 101: 1731–1737)

Colorectal cancer (CRC) is one of the most common causes of cancer death in the world.[1] Despite improved surgical techniques, adjuvant/neoadjuvant chemotherapy, and molecular target therapy, patients with progressive disease still have poor clinical outcomes. The 5-year survival rate for patients with localized CRC is about 90%, whereas the rate for patients with regional and distal metastases is only 60%, and 10%, respectively.[1,2] New markers for cancer prognosis and therapeutic options targeting invasion and metastasis are needed to improve treatment of CRC.

Mutations in both alleles of the adenomatous polyposis coli (*APC*) gene are associated with >70% of human intestinal carcinomas.[3] This mutation results in the accumulation of β-catenin in the nucleus and aberrant activation of a Wnt target gene program that initiates the transformation of intestinal epithelial cells.[4–7] Patients with familial adenomatous polyposis (FAP) carry the APC mutation, which develops multiple colonic adenomas, indicating that loss of the APC ''gatekeeper'' function is associated with the earliest stages of intestinal tumorigenesis.[3]

Recent studies suggest that leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), also known as GPR49, is a target of Wnt signaling.[7–9] LGR5 is a member of the G-protein-coupled receptor (GPCR) family comprising proteins with seven transmembrane domains. GPCRs function as receptors for various classes of ligand, including peptide hormones and chemokines.[10] To date, the ligand for, and function of, LGR5-related signaling remain unclear. A recent study showed that LGR5-null mice exhibited neonatal lethality, characterized by ankyloglossia and gastrointestinal distension.[11] It was also reported that LGR5 is a marker for stem cells in the small intestine and colon,[12] and in the hair follicles of mice.[13] Therefore, LGR5 may play a crucial role in the biological function of stem cells. As we reported previously, LGR5 is overexpressed in basal cell cancers (BCCs) and hepatocellular carcinomas (HCCs).[9,14] LGR5 expression is down-regulated in colon cancer cell lines as a result of Wnt signaling suppression, and is up-regulated in intestinal adenomas from humans with FAP and mice with the germline APC mutation.[7,8] Therefore, LGR5 may be involved in colorectal carcinogenesis as a Wnt target gene. We specifically analyzed LGR5 gene expression using a panel of cancer cell lines and colorectal surgical specimens.

Materials and Methods

Patients and samples. Surgical resections were performed on 395 patients with CRC at Keio University Hospital, Japan, between December 2005 and April 2008. Curative operations were performed on 89 patients with pT3/T4 and stage II/III CRC. From the 89 patients, we selected 50 patients: 25 cases each positive and negative for lymph node metastases. Surgically resected specimens of cancerous tissue and matched normal mucosal tissue were obtained from the 50 patients for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. All samples were taken from surgically resected material, immediately frozen in liquid nitrogen and stored at −80°C. Adequate lymph node dissection was performed in all 50 cases and a pathological diagnosis was made according to the TMN classification. These 50 patients comprised 27 males and 23 females; average age 66.0 years (range, 48–87 years). Clinicopathologically, there were no significant differences between the two groups except for lymphatic invasion and venous invasion (Table 1). None of the 50 patients had synchronous cancers, ulcerative colitis, or FAP and none had received pre-operative chemotherapy or radiation therapy. These 50 cases were evaluated for the association between LGR5 expression and clinicopathological findings. Tissue specimens were also obtained from seven patients with sporadic colorectal adenomas. Specimens were collected, stored, and analyzed, as described previously. The study was approved by the ethics committee of the School of Medicine, Keio University.

Cell culture. Thirty-seven cell lines derived from colon cancers (COLO 201, COLO 205, HT-29, HCT-15, SW1116, SW480, SW620, LoVo, WiDr, Caco-2, and HCT 116), HCCs (Hep G2, PLC/PRF/5, KIM-1, KYN-2, and Li7), ovarian cancers (ES-2, TOV-21G, RMG I, RMG II, RMG V, TOV-112D, RMUG-S, RMUG-L, HTOA, and KF 11), and lung cancers (SK-LU-1, Calu-3, A549, PC9, H1650, H1975, SK-MES-1, SW 900, H69, H146, and H889), were used. Details of the colon

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Table 1. Patient characteristics of 50 cases of pT3/T4 colorectal cancer

	Total (%)	Lymph node metastasis		*P*-value
		Negative (%)	Positive (%)	
		25 (50)	25 (50)	
Age				
≥65 years	27 (54)	17 (63)	10 (37)	0.0877*
<65 years	23 (46)	8 (35)	15 (65)	
Gender				
Female	23 (46)	15 (65)	8 (35)	0.0877*
Male	27 (54)	10 (37)	17 (63)	
Tumor size				
≥50 mm	21 (42)	12 (57)	9 (43)	0.5672*
<50 mm	29 (58)	13 (45)	16 (55)	
Tumor invasion†				
pT3	45 (90)	24 (53)	21 (47)	0.3487*
pT4	5 (10)	1 (20)	4 (80)	
Tumor location				
Right side‡	25 (50)	15 (60)	10 (40)	0.2578*
Left side§	25 (50)	10 (40)	15 (60)	
Differentiation				
Well	6 (12)	5 (83)	1 (17)	0.1195¶
Moderate	43 (86)	19 (44)	24 (56)	
Mucinous	1 (2)	1 (100)	0 (0)	
Lymphatic invasion				
Negative	17 (34)	15 (88)	2 (12)	0.0002*
Positive	33 (66)	10 (30)	23 (70)	
Vascular invasion				
Negative	15 (30)	15 (100)	0 (0)	<0.0001*
Positive	35 (70)	10 (29)	25 (71)	

*Fisher's extract test; †tumor invasion, TNM classification by International Union Against Cancer (UICC) was adopted. ‡Right side, from cecum to transverse colon; §left side, from descending colon to rectum; ¶χ^2-test. There was no significant difference between lymph node metastasis and clinicopathological findings except for lymphatic and vascular invasion.

Table 2. Characteristics of colon cancer cell lines

Cell line	Gender	Age	Site	Dukes' stage
LoVo	M	56	Colon, distant lymph node	C
Caco-2	M	72	Colon	NA
SW480†	M	50	Colon	B
SW620†	M	51	Colon, metastatic lymph nodule	C
COLO 205‡	M	70	Cecum, ascitis	D
COLO 201‡	M	70	Cecum, ascitis	D
HT-29	F	44	Colon	NA
WiDr	F	78	Colon	NA
HCT-15	M	NA	Colon	C
SW1116	M	73	Colon	A
HCT 116	M	NA	Colon	NA

†,‡Indicate origin from same patient. NA, not available.

cancer cell lines are shown in Table 2. All cell lines were obtained from ATCC (Manassas, VA, USA) except for PC9 (Immuno-Biological Laboratories, Takasaki, Japan), and KIM-1, KYN-2, Li7, RMG I, RMG II, RMG V, RMUG-S, RMUG-L, HTOA, and KF.[9,15–17] All cell lines, except SK-LU-1, Calu-3, A549, PC9, H1650, H1975, SK-MES-1, and SW 900, were routinely maintained at 37°C with 5% CO_2 in RPMI-1640 containing 10% FBS. SK-LU-1, Calu-3, A549, PC9, H1650, H1975, SK-MES-1, and SW 900 were routinely maintained at 37°C with 5% CO_2 in DMEM containing 20% FBS.

Quantitative RT-PCR. Total RNA was isolated from cultured cells and CRC tissues using RNeasy Mini Kits (Qiagen, Tokyo, Japan), and RNAiso (Takara Bio, Shiga, Japan), with a slight modification of the DNase treatment. RNA quality was assessed in the RNA 6000 Nano LabChip using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). cDNA was synthesized using the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio). Quantitative RT-PCR analysis was performed on a Thermal Cycler Dice Real Time System using SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio). The primer sequences for GAPDH were 5′-ATCATCCCTGCCTCTACTGG-3′ and 5′-TTTCTAGACGGCAGGTCAGGT-3′, and those for LGR5 were 5′-TCTCAGCCATGGTGAACAA-3′ and 5′-TAGCGAATCACCAGGAAGGT-3′. Fold-induction values were calculated using the $2^{-\Delta\Delta Ct}$ method and LGR5 expression was normalized to GAPDH. All experiments were performed in triplicate and repeated in at least three separate experiments; representative data from these experiments are presented.

Immunohistochemical analyses. For immunohistochemistry, normal intestinal mucosal tissues were analyzed. Formalin-fixed and paraffin-embedded serial sections were incubated with Ki-67 mouse monoclonal antibody (1 : 200; Dako, Glostrup, Denmark) and chromogranin A rabbit polyclonal antibody (1 : 4000; Dako) for 1 h at room temperature for detection of proliferating cells and enteroendocrine cells, respectively, in intestinal crypts. Primary antibody binding was visualized using an ImmPRESS Anti-Mouse Ig Kit (Vector Laboratories, Burlingame, CA, USA) for Ki-67, and swine antirabbit secondary antibody (Dako) for chromogranin A, with diaminobenzidine as the chromogen. The sections were counterstained with hematoxylin and mounted.

***In situ* hybridization.** Tissues were fixed in 10% formalin and embedded in paraffin, and 5-μm sections were cut and placed on poly-L-lysine-coated slides. Digoxigenin-labeled LGR5 sense and antisense probes were generated from a 359-bp fragment of LGR5 (corresponding to nucleotides 1730–2088, GenBank ID NM_003667) using the DIG-RNA labeling Kit (SP6/T7) (Roche Diagnostics, Mannheim, Germany). *In situ* hybridization (ISH) was performed according to the following protocol. De-paraffinized sections were treated with proteinase K (10 μg/mL) for 9 min at 37°C, and acetylated and prehybridized in hybridization solution (50% formamide, 5× SSC, 1% SDS, 0.05 mg/mL yeast RNA, 0.05 mg/mL heparin) for 1 h at 65°C. Hybridization was performed for 16 h at 70°C with hybridization solution containing the probe (1 ng/μL). Post-hybridization washes were performed as follows: 3× washes with 50% formamide and 4× SSC for 15 min at 70°C, 3× washes with 50% formamide and 2× SSC for 15 min at 65°C and 3× washes with 0.5 mg/mL levamisol in Tris-buffered saline-Tween-20 (TBST) for 10 min at room temperature. Following the washes, blocking was performed with 1% sheep serum in TBST containing 0.5 mg/mL levamisol for 30 min. Sections were incubated with anti-digoxigenin-alkaline phosphatase (Roche Diagnostics), diluted 1 : 3000 in TBST for 2 h at room temperature, followed by blocking. Sections were visualized using BM Purple AP substrate (Roche Diagnostics), followed by equilibration in NTMT (0.1 M Tris-HCl [pH 9.5], 50 mM $MgCl_2$, 0.1 M NaCl, and 0.1% Tween-20).

Statistical analysis. Paired *t*-tests were used to analyze differences in LGR5 expression between CRCs and the corresponding colonic mucosal tissues. Unpaired *t*-tests were used to analyze the association between LGR5 expression and clinicopathological parameters. StatView (version 5.0) software (Abacus Concepts, Berkeley, CA, USA) was used and *P*-values <0.05 were considered significant.

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Results

Overexpression of LGR5 in colon cancer cell lines. To evaluate the expression of LGR5 in CRC compared with other types of cancers, qRT-PCR analysis was performed on a panel of representative cancer cell lines derived from different organs: 11 colon cancer cell lines, five HCC cell lines, 10 ovarian cancer cell lines, and 11 lung cancer cell lines (Fig. 1). LGR5 expression in each of the cell lines was normalized to the average LGR5 expression in the 37 cell lines. LGR5 mRNA was overexpressed in colon cancer cell lines (5/11), whereas LGR5 overexpression was rare in the cell lines derived from other cancers, only occurring in Hep G2 among the HCCs (1/5) and in H889 among the lung cancer cell lines (1/11). Colon cancer cell lines more frequently displayed elevated mRNA levels for LGR5 compared with other cell lines. Elevated expression of LGR5 was observed in all four colon cancer cell lines derived from metastatic sites (LoVo, SW620, COLO 201, and COLO 205) but in only one of seven derived from primary sites ($P = 0.0288$) (Fig. 1). The mean expression of LGR5 was three times higher in cell lines derived from metastatic sites compared with those derived from primary sites.

Overexpression of LGR5 in colorectal cancers and adenomas. LGR5 expression was assessed by qRT-PCR in surgical specimens from stage II/III CRCs. As shown in Figure 2(a), LGR5 expression was markedly elevated in most cancer tissues compared with normal mucosal tissues. Of the 50 cases, 35 (70%) showed LGR5 expression levels more than three times higher than those of the matched normal mucosal tissues.

LGR5 expression in sporadic adenomas was also investigated. As shown in Figure 2(b), all seven colorectal adenomas showed marked up-regulation of LGR5 compared with normal colonic mucosa. The mean expression of LGR5 in CRCs and adenomas was significantly higher than that in normal mucosa (0.039 ± 0.069 *vs.* 0.003 ± 0.002, $P = 0.0005$ and 0.016 ± 0.005 *vs.* 0.002 ± 0.001, $P = 0.0003$, respectively) (Fig. 2c). Variation in LGR5 expression was observed among cancer tissues, whereas adenoma tissues and normal mucosal tissues showed comparatively stable LGR5 expression.

Localization of LGR5-expressing cells in colorectal tumors and normal intestinal mucosa. Since a specific antibody to LGR5 for immunohistochemical analysis was not available, ISH was performed to confirm LGR5 expression in CRC cases (Fig. 3a–f). LGR5 mRNA was detected in the tumor cells in the colon cancer cases. However, LGR5 was not expressed in the stromal tissue surrounding the tumors. LGR5 mRNA was diffusely expressed in the entire tumor, including the tumor edge and invasive front (Fig. 3a–c). Similar results were obtained in adenomas (Fig. 3g–i).

In contrast, no clear positive signals were observed in normal mucosal tissue, except for the crypt base cells of the small intestine (Fig. 4c–f) and colon (data not shown). Careful observation showed that crypt base columnar (CBC) cells between Paneth cells expressed LGR5 mRNA (arrowheads, Fig. 4d). Immunohistochemistry on serial sections showed that Ki-67-positive cells were mainly the transamplifying cells and small numbers of CBC cells in the intestinal crypt (Fig. 4g,h). Figure 4(h) shows Ki-67 staining of nuclei in LGR5-positive CBC cells. LGR5-positive cells may have low proliferative activity compared with transamplifying cells. Chromogranin A-positive enteroendocrine cells (arrow, Fig. 4j) were occasionally detected in the crypt base. However, LGR5 positive cells were negative for chromogranin A.

Correlation of LGR5 overexpression with malignant potential. The clinicopathological significance of variable expression of LGR5 in CRC was evaluated. When LGR5 expression normalized to that of matched normal mucosa, LGR5 expression was significantly greater in cases with lymph node metastasis, lymphatic invasion, vascular invasion and pT4 stage tumors ($P = 0.0004$, $P = 0.01$, $P = 0.0206$ and $P = 0.0024$, respectively) (Fig. 5). Moreover, LGR5 overexpression correlated significantly with the number of lymph node metastases ($P < 0.0001$), and LGR5 expression in stage IIIC tumors was approximately two-fold higher than in stage IIIB tumors (26.6 ± 24.5 *vs.* 14.0 ± 13.0, $P = 0.0245$) (Fig. 6a,b). However, LGR5 expression did not correlate with the age or gender of the patient, or with tumor differentiation, location, or size (Fig. 5).

Discussion

It is thought that the adenoma–carcinoma sequence underlies the development of CRC, and that the progression of

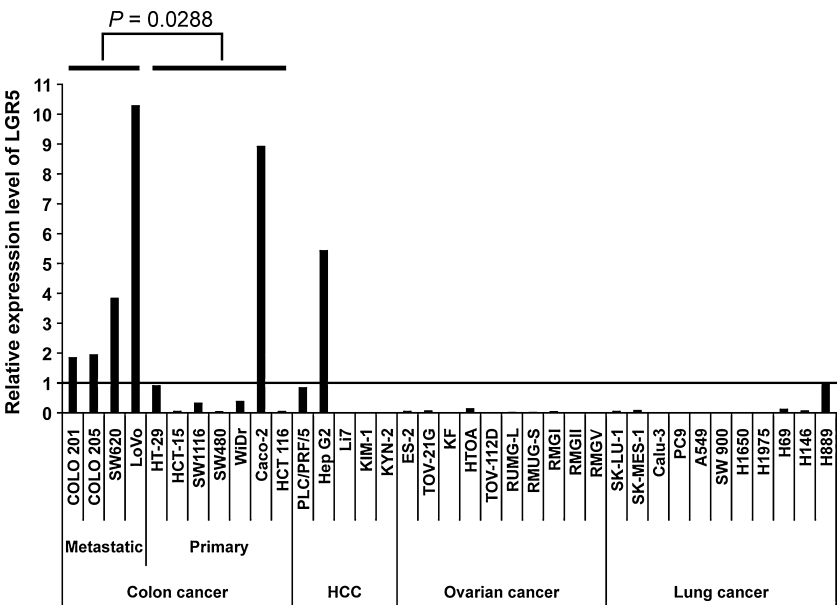

Fig. 1. Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) expression in a variety of cancer cell lines. LGR5 mRNA expression was evaluated by qRT-PCR analysis in 11 colon cancer cell lines, five hepatocellular carcinoma (HCC) cell lines, 10 ovarian cancer cell lines, and 11 lung cancer cell lines. LGR5 expression was normalized to that of GAPDH. The average LGR5 expression in the 37 cell lines was set at 1 (bold line). LGR5 mRNA was frequently overexpressed in colon cancer cell lines (5/11) compared with other cancer cell lines (only Hep G2 for HCC, H889 for lung cancer, and none of the ovarian cancers). All colon cancer cell lines derived from metastatic sites showed overexpression of LGR5.

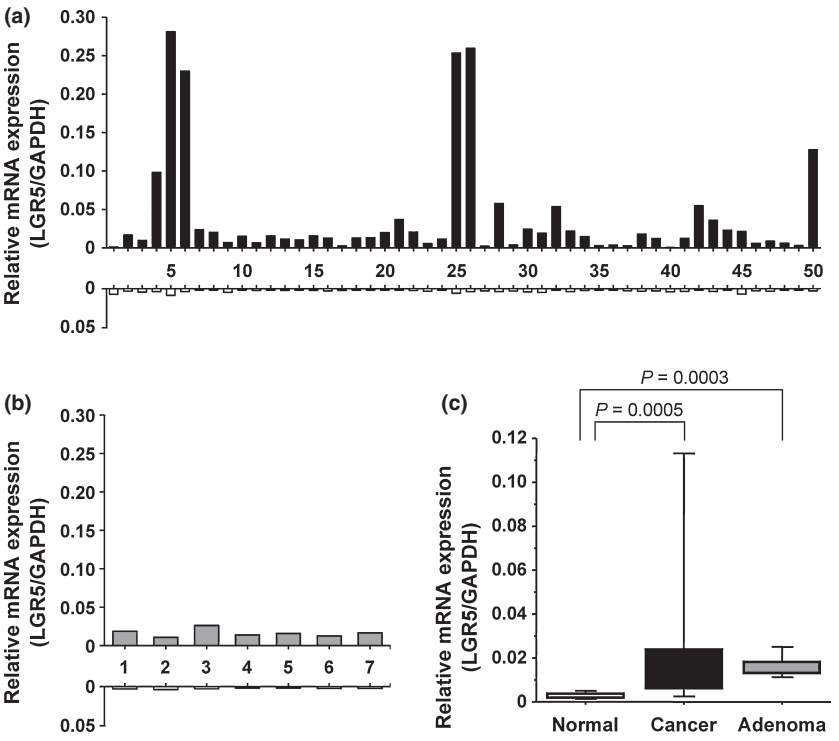

Fig. 2. Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) expression in colorectal tumor tissue and matched normal mucosa. LGR5 mRNA expression was estimated by qRT-PCR and was normalized to that of GAPDH. (a) LGR5 expression, in 50 cases of colorectal cancer (CRC) and matched normal colorectal mucosa, was evaluated (upper column, mRNA expression in tumors; lower column, mRNA expression in normal colonic mucosa). The majority of CRCs (70%) showed a more than three times greater expression of LGR5 compared with matched normal mucosa. (b) In seven sporadic colorectal adenomas, LGR5 expression was significantly higher than in matched normal mucosa. (c) Mean (SD) mRNA expression levels for LGR5 in CRC, normal mucosa, and colorectal adenoma. LGR5 mRNA expression was approximately 13-fold higher in CRCs as compared with normal mucosa, and LGR5 expression showed marked variation in cancers as compared with adenomas.

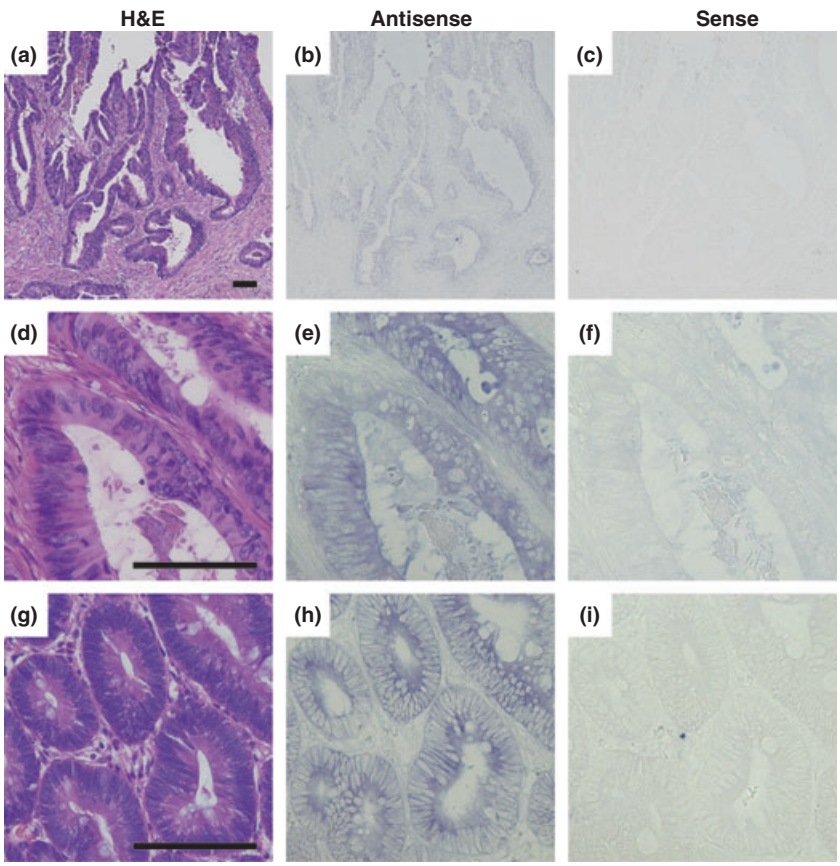

Fig. 3. *In situ* hybridization of Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) mRNA in colorectal cancers (CRCs) and adenomas. De-paraffinized sections were stained with H&E (a,d,g), and hybridized with antisense (b,e,h) or sense (c,f,i) probes for LGR5. Scale bars, 100 μm. Expression of the antisense signal coincided specifically with cancer cells (a–c, ×40; d–f, ×200) and adenoma cells (g–i, ×200). The LGR5 signal was diffusely expressed over the entire cancer tissue.

adenoma to carcinoma is a slow process driven by a linear and stepwise series of genetic alterations involving key genes such as APC, KRAS, and TP53.[18] Biallelic mutation of APC, resulting in aberrant activation of Wnt signaling, was observed frequently in CRC.[3] Previous studies showed that LGR5 was overexpressed in BCCs[14] and HCCs, with activa-

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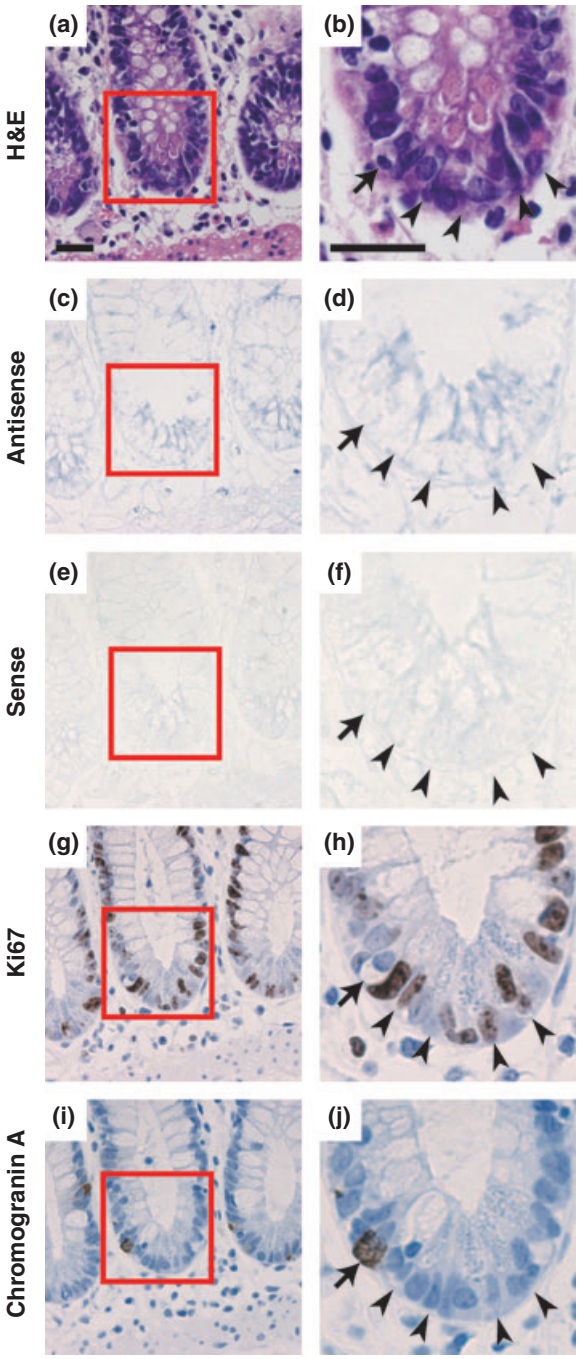

Fig. 4. *In situ* hybridization of Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) mRNA in normal epithelium of the small intestine. De-paraffinized serial sections were stained with H&E (a,b), hybridized with antisense (c,d) and sense (e,f) probes for LGR5, and immunolabeled for Ki-67 (g,h) and chromogranin A (i,j). Scale bars, 25 μm. The enlarged crypt epithelium is shown on the right (b,d,f,h,j). Expression of antisense signal for LGR5 coincided specifically with four crypt base columnar (CBC) cells (arrowheads) between Paneth cells in the crypt of the small intestine (×400) (d). Nuclei of the CBC cells were lightly stained for Ki-67 (arrowheads, h). The cells hybridized with antisense probe did not express chromogranin A (arrow, j).

tion of Wnt signaling by a β-catenin mutation.[9] Moreover, from recent findings, it was suggested that LGR5 may be involved in colorectal carcinogenesis as a Wnt target gene.[7,8] Barker *et al.*[12] performed lineage-tracking experiments in mice and showed that *Lgr5*-expressing CBC cells at crypt positions 1–4 fulfilled all the criteria for putative intestinal stem cells. They also showed that selective transformation of Lgr5-enhanced green fluorescent protein-positive stem cells after knockout of Apc, and efficiently induced neoplasia in both the small intestine and colon of mice.[19] The present study has shown that LGR5 was markedly overexpressed in the majority of advanced CRCs (70%) compared with normal mucosal tissue, suggesting that overexpression of LGR5 is a common feature of CRC. Moreover, the ISH signal for LGR5 was detected in the CBC cells, which are thought to be intestinal stem cells, in both small intestine and colon. LGR5 was also detected in adenoma and CRC cells. These findings suggest that LGR5 may be a marker for intestinal stem cells, as well as for colorectal adenoma and carcinoma in humans. Taken together with recent findings,[12,19] the present data suggests that the origin of human CRC may be intestinal stem cells expressing LGR5.

Interestingly, LGR5 expression was higher in colon cancer cell lines derived from metastatic tumors compared with those derived from primary tumors. Therefore, lymph node metastasis positive and negative cases of CRC were compared. Marked variation in LGR5 expression was observed among CRC samples, compared with the samples of normal mucosa and adenoma samples. Increased expression of LGR5 in CRCs was significantly correlated with lymph node metastases of advanced CRCs. These results suggest that LGR5 expression may be associated with the malignant potential of CRC. Since the mechanism of LGR5 involved in colorectal tumorigenesis remains unclear, further studies are needed to elucidate the functions of LGR5. However, we show LGR5 as a promising prognostic marker in management of cancer patients, because patients with lymph node metastases have poor clinical outcomes after curative surgery.[1,2] Thus, the intensity of LGR5 expression could be an indicator of the need for postsurgical adjuvant therapy in CRC patients.

LGR5 was originally isolated as a leucine-rich, orphan G-protein-coupled, seven-transmembrane receptor. LGR5 is a member of the glycoprotein hormone receptor subfamily that includes thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) receptors.[20,21] The GPCR families are major targets for pharmaceutical development.[22] Thyroid-stimulating hormone (TSH) suppression following thyroidectomy has been reported to reduce the rate of recurrence of thyroid cancer.[23,24] Luteinizing hormone-releasing hormone (LHRH) agonists, whose receptors are also a member of the GPCR family, provide an additional class of agents for the treatment of premenopausal women with hormone receptor-positive breast cancer.[25,26] Luteinizing hormone-releasing hormone (LHRH) agonist therapy has also been the mainstay of treatment for advanced prostate cancer.[27] Orphan GPCRs are potential new drug targets and are currently being investigated for their potential usefulness in the treatment of debilitating diseases, including obesity, cardiovascular disease, inflammation, and cancer.[28–31] The present study suggests that LGR5, a member of the GPCR family, plays an important role in colorectal tumorigenesis, and these findings are supported by recent studies.[7,8,19] Therefore, LGR5 may be a promising therapeutic target for the management of patients with CRC.

In conclusion, LGR5, a putative intestinal stem cell marker, was frequently overexpressed in adenomas and CRCs. These results suggest that LGR5 plays important roles, not only in early events, but also in late events (invasion and metastasis) in colorectal tumorigenesis. Therefore, LGR5 may be a novel prognostic marker and a candidate therapeutic target in CRC.

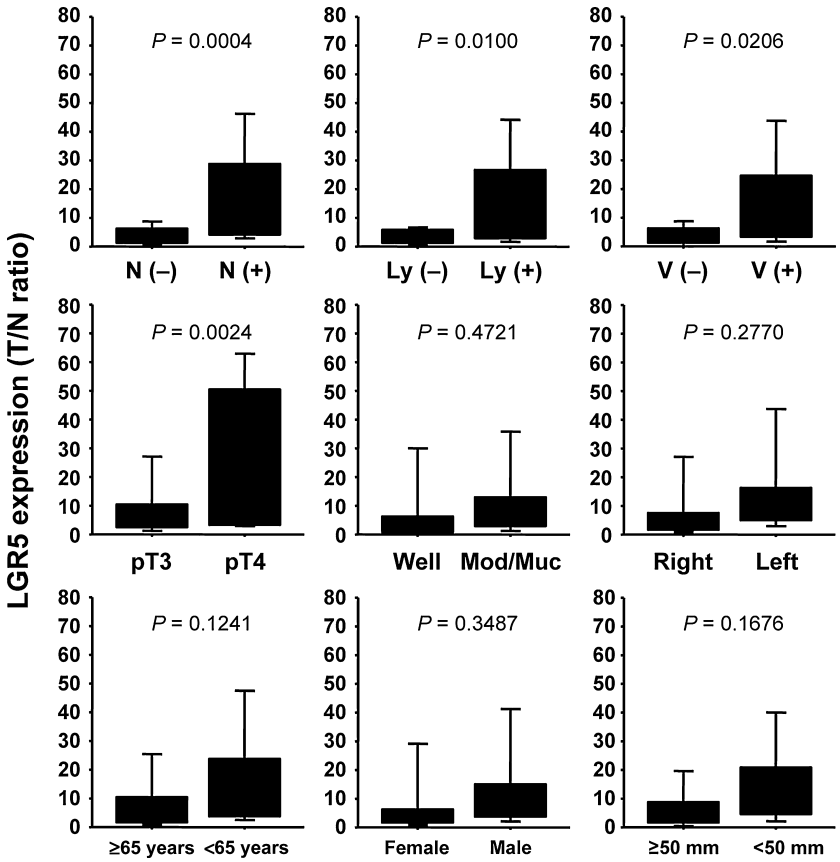

Fig. 5. Correlation between Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) mRNA expression and clinicopathological findings in colorectal cancers (CRCs). LGR5 expression in CRCs was divided by that in matched normal mucosa (T/N ratio). Lymph node metastasis (N)-positive cases, those with lymphatic invasion (Ly) and vascular invasion (V), and pT4 cases, showed significantly higher LGR5 expression compared with other CRCs. LGR5 expression did not correlate with other clinicopathological findings such as age and gender of the patient, and tumor differentiation, location, and size.

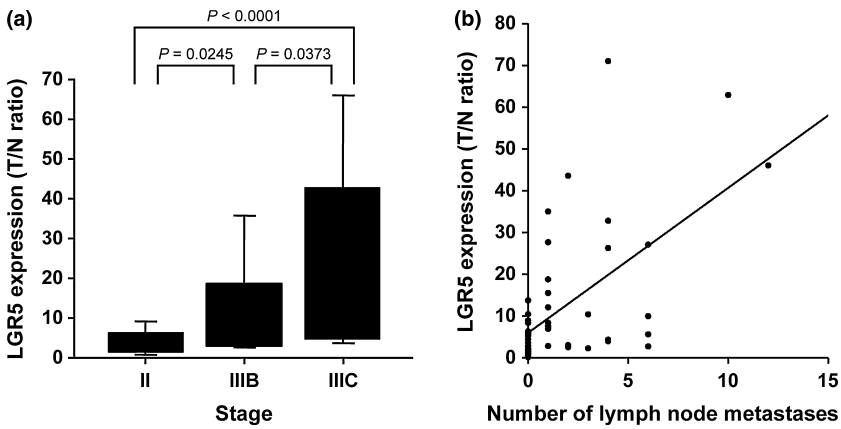

Fig. 6. Association between Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) expression and malignant potential of colorectal cancer (CRC). LGR5 expression in CRCs was divided by that in matched normal mucosa (T/N ratio). (a) Mean LGR5 expression in stages II (lymph node metastasis 0), IIIB (lymph node metastases 1–3) and IIIC (lymph node metastases ≥4). LGR5 expression increased stepwise with tumor stage progression. (b) LGR5 expression correlated positively with the number of lymph node metastases ($r = 0.59$, $P < 0.0001$).

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Disclosure Statement

The authors have no conflict of interest.

References

1 Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007; **57**: 43–66.
2 O'Connell JB, Maggard MA, Ko CY. Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. *J Natl Cancer Inst* 2004; **96**: 1420–5.
3 Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159–70.
4 Korinek V, Barker N, Morin PJ *et al.* Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC−/− colon carcinoma. *Science* 1997; **275**: 1784–7.

doi: 10.1111/j.1349-7006.2010.01571.x

5 Morin PJ, Sparks AB, Korinek V *et al.* Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 1997; **275**: 1787–90.
6 van de Wetering M, Sancho E, Verweij C *et al.* The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 2002; **111**: 241–50.
7 Van der Flier LG, Sabates-Bellver J, Oving I *et al.* The intestinal Wnt/TCF signature. *Gastroenterology* 2007; **132**: 628–32.
8 Segditsas S, Sieber O, Deheragoda M *et al.* Putative direct and indirect Wnt targets identified through consistent gene expression changes in APC-mutant intestinal adenomas from humans and mice. *Hum Mol Genet* 2008; **17**: 3864–75.
9 Yamamoto Y, Sakamoto M, Fujii G *et al.* Overexpression of orphan G-protein-coupled receptor, Gpr49, in human hepatocellular carcinomas with beta-catenin mutations. *Hepatology* 2003; **37**: 528–33.
10 Schoneberg T, Schultz G, Gudermann T. Structural basis of G protein-coupled receptor function. *Mol Cell Endocrinol* 1999; **151**: 181–93.
11 Morita H, Mazerbourg S, Bouley DM *et al.* Neonatal lethality of LGR5 null mice is associated with ankyloglossia and gastrointestinal distension. *Mol Cell Biol* 2004; **24**: 9736–43.
12 Barker N, van Es JH, Kuipers J *et al.* Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007; **449**: 1003–7.
13 Jaks V, Barker N, Kasper M *et al.* Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nat Genet* 2008; **40**: 1291–9.
14 Tanese K, Fukuma M, Yamada T *et al.* G-protein-coupled receptor GPR49 is up-regulated in basal cell carcinoma and promotes cell proliferation and tumor formation. *Am J Pathol* 2008; **173**: 835–43.
15 Aoki D, Suzuki N, Susumu N *et al.* Establishment and characterization of the RMG-V cell line from human ovarian clear cell adenocarcinoma. *Hum Cell* 2005; **18**: 143–6.
16 Ishiwata I, Ishiwata C, Soma M, Nozawa S, Ishikawa H. Characterization of newly established human ovarian carcinoma cell line – special reference of the effects of cis-platinum on cellular proliferation and release of CA125. *Gynecol Oncol* 1987; **26**: 340–54.
17 Suzuki N, Aoki D, Oie S *et al.* A novel retinoid, 4-[3,5-bis (trimethylsilyl) benzamido] benzoic acid (TAC-101), induces apoptosis of human ovarian carcinoma cells and shows potential as a new antitumor agent for clear cell adenocarcinoma. *Gynecol Oncol* 2004; **94**: 643–9.
18 Vogelstein B, Fearon ER, Hamilton SR *et al.* Genetic alterations during colorecta-tumor development. *N Engl J Med* 1988; **319**: 525–32.
19 Barker N, Ridgway RA, van Es JH *et al.* Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* 2009; **457**: 608–11.
20 Hsu SY, Liang SG, Hsueh AJ. Characterization of two LGR genes homologous to gonadotropin and thyrotropin receptors with extracellular leucine-rich repeats and a G protein-coupled, seven-transmembrane region. *Mol Endocrinol* 1998; **12**: 1830–45.
21 McDonald T, Wang R, Bailey W *et al.* Identification and cloning of an orphan G protein-coupled receptor of the glycoprotein hormone receptor subfamily. *Biochem Biophys Res Commun* 1998; **247**: 266–70.
22 Brink CB, Harvey BH, Bodenstein J, Venter DP, Oliver DW. Recent advances in drug action and therapeutics: relevance of novel concepts in G-protein-coupled receptor and signal transduction pharmacology. *Br J Clin Pharmacol* 2004; **57**: 373–87.
23 Mazzaferri EL, Jhiang SM. Long-term impact of initial surgical and medical therapy on papillary and follicular thyroid cancer. *Am J Med* 1994; **97**: 418–28.
24 McGriff NJ, Csako G, Gourgiotis L, Lori CG, Pucino F, Sarlis NJ. Effects of thyroid hormone suppression therapy on adverse clinical outcomes in thyroid cancer. *Ann Med* 2002; **34**: 554–64.
25 Cuzick J, Ambroisine L, Davidson N *et al.* Use of luteinising-hormone-releasing hormone agonists as adjuvant treatment in premenopausal patients with hormone-receptor-positive breast cancer: a meta-analysis of individual patient data from randomised adjuvant trials. *Lancet* 2007; **369**: 1711–23.
26 Schmid P, Untch M, Kosse V *et al.* Leuprorelin acetate every-3-months depot versus cyclophosphamide, methotrexate, and fluorouracil as adjuvant treatment in premenopausal patients with node-positive breast cancer: the TABLE study. *J Clin Oncol* 2007; **25**: 2509–15.
27 Moreau JP, Delavault P, Blumberg J. Luteinizing hormone-releasing hormone agonists in the treatment of prostate cancer: a review of their discovery, development, and place in therapy. *Clin Ther* 2006; **28**: 1485–508.
28 Bennett T, Gardiner SM. Fostering orphan receptors: an indispensable role for integrative, *in vivo*, haemodynamic studies. *Curr Opin Pharmacol* 2003; **3**: 140–5.
29 Katugampola S, Davenport A. Emerging roles for orphan G-protein-coupled receptors in the cardiovascular system. *Trends Pharmacol Sci* 2003; **24**: 30–5.
30 Shaaban S, Benton B. Orphan G protein-coupled receptors: from DNA to drug targets. *Curr Opin Drug Discov Devel* 2001; **4**: 535–47.
31 Willson TM, Moore JT. Genomics versus orphan nuclear receptors – a half-time report. *Mol Endocrinol* 2002; **16**: 1135–44.