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Note

Intensive Immunofluorescence Staining Methods for Low Expression Protein: Detection of Intestinal Stem Cell Marker LGR5

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Leucine-rich repeat-containing G-protein coupled receptor 5, or LGR5, is a molecule that recognizes stem cells in multiple organs and also in colon cancer. Previously, we have developed monoclonal antibodies specific to LGR5 protein that can be used for immunofluorescence staining, but because a very low level of LGR5 protein is expressed, the visualization technique needed to be enhanced. To develop procedures to detect LGR5 protein in various specimens by immunofluorescence staining, we evaluated the Alexa-labeled streptavidin biotin (LSAB), the Qdot, and the tyramide methods. The detection sensitivity was highest in the tyramide method followed by the Qdot method, whereas subcellular localization of the protein was most clear in the Qdot method, because the Qdot method gave a high S/N ratio that could show a low background. Thus, the tyramide method is superior to the Q-dot method for intensifying the signal of a low expression protein, and the Qdot method is superior to the tyramide method for identifying the subcellular localization of the target protein. The results of the present study will be helpful in providing more insight into the pathophysiological roles of LGR5-positive cancer stem cells and in developing therapeutic approaches for targeting cancer stem cells.

Key words: LGR5, immunofluorescence, Alexa, Qdot, tyramide

I. Introduction

Leucine-rich repeat-containing G-protein coupled receptor 5, or LGR5, is expressed in intestinal stem cells [2–4]. Lineage tracing with mice, in which the LGR5 gene promoter was linked to the lacZ gene, demonstrated that all epithelial cells of the intestinal villi stemmed from the LGR5-positive cells in the crypt, further confirming that the LGR5-positive cells are intestinal stem cells. Genetic transformation of LGR5-positive cells in normal intestine has shown their potential as tumor-initiating cells [4]. In one example, an organoid generated from normal human intestine including LGR5-positive cells and harbored mutations in the tumor suppressor genes and oncogenes resulted in the formation and expansion of adenoma organoids [11]. Furthermore, LGR5 was shown to be expressed in human colon cancer stem cells [4, 10, 12, 18, 19]. We also established human colon cancer stem cell lines and found that they expressed LGR5 [8]. They continued to expand *in vitro*, but upon exposure to anticancer agents, they stopped proliferating and became LGR5-negative. After removal of the anticancer agent, they proliferated again, and expression of LGR5 was restored, which indicates the ability of cancer stem cells to transition between proliferating and

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arresting phases.

Previously, we reported the generation of monoclonal anti-LGR5 antibodies [8], because there was no specific antibody that was available for immunohistochemical staining (IHC) [1, 7]. These antibodies are highly specific to LGR5 and do not react with LGR4 and LGR6, which are highly homologous to LGR5. Using these antibodies, we confirmed that, although rare, LGR5-positive cancer stem cells are present in human colon cancer tissues. However, the level of LGR5 protein expressed in cancer stem cells was very low [17], and therefore, visualization must be enhanced to be able to detect the protein by IHC.

The successful detection of LGR5 protein mentioned earlier was in patient-derived specimens that had been processed under well-controlled laboratorial conditions. It is well known that visualization is enhanced by the quality of the specimens. In our retrospective analysis, however, appropriate methods of enhancement needed to be identified and applied to the archived samples created by conventional method with 10% neutral buffer formalin fixation. One method is offered by the new fluorescent dyes that have been developed [5, 9, 21]. In addition, since the quality of fluorophores has been highly refined by increasing their brightness, the range at which low expression proteins can be detected has expanded, and researchers are able to perform more precise quantitative analyses [5, 21, 22]. In this study, we evaluated those methods of intensifying the visualization signals to detect low expression LGR5 protein.

II. Materials and Methods

Study design

We tried three immunofluorescence staining methods to detect LGR5: 1) Alexa Fluor® (Life Technologies, CA, USA), a widely used type of fluorophore, 2) Qdot® (Life Technologies), a high-brightness fluorophore, and 3) Tyramide Signal Amplification™, in which high sensitivity is achieved by an accumulated deposition of labeling dye. We decided to apply immunofluorescence staining methods because it became clear that specific reaction could not been detected in bright field by visualization using 3,3'-diaminobenzidine as a result of a preliminary study. The detection level of each method was analyzed in three cell lines that have different expression levels of LGR5. The cell lines are 1) an established colon cancer stem cell line (LGR5+CSC), in which all of the cells express LGR5, 2) a commercially available colon cancer cell line, LOVO (American Type Culture Collection, Manassas, VA, USA), in which some of the cells express LGR5, and 3) a commercially available colon cancer cell line, HCT116 (American Type Culture Collection), which does not express LGR5 [8].

Next, we used the highly sensitive immunofluorescence staining methods to detect LGR5 in tissue blocks from the intestine of a normal cynomolgus monkey and from a human colorectal adenoma region.

Anti-LGR5 antibody

We previously generated a new antibody specific to LGR5 [8]. Briefly, an anti-LGR5 monoclonal antibody, 2U2E-2, was obtained by protein immunization. BALB/c mice were immunized subcutaneously with the LGR5-Fc protein once a week for 2 weeks. Three days before cell fusion, mice were injected intravenously. Hybridomas were generated and clone 2U2E-2 was selected by testing the antibody with ELISA using the LGR5-Fc protein. Specificity of the antibody was tested by IHC and flow cytometry using CHO cells that express highly related proteins LGR4, LGR5, or LGR6.

Cell blocks from cultivated cancer cells

LGR5+CSCs were generated by the method that was previously described [8]. Briefly, a single cell suspension of cancer cells was prepared from a patient-derived xenograft by mincing the tissues, then incubated with collagenase/dispase (Roche, Basel, Switzerland) and DNase I (Roche) and finally suspended in lysis buffer (BD Biosciences, CA, USA). The cells were cultured in culture flasks treated with with polystyrene (BD Biosciences) in a stem cell medium. LOVO and HCT116 cell lines were cultured by standard methods. In addition, to obtain drug-resistant LGR5-negative cells (LGR5-CSCs) as a negative control, LGR5+CSCs were treated with 10 μg/ml irinotecan for 3 days.

The cultured cells were removed from the culture dish after fixation by 4% paraformaldehyde (PFA) or 10% neutral buffer formalin for 2 hr. After washing by phosphate buffered saline, the cells were mixed into agarose gel. After the agarose gel solidified, the cell pellets were embedded into paraffin by the AMeX method [15, 16].

Preparation of paraffin sections from a tissue block of a normal cynomolgus monkey

To study the difference between enhancing methods using a block made under laboratorial conditions, a paraffin block of normal intestine was selected from our panel of cynomolgus monkey tissues, and paraffin sections for immunofluorescence staining were prepared. The tissue panel blocks were fixed in 4% PFA at 4°C for 16 to 24 hr, and paraffin-embedded tissue blocks were prepared by the AMeX method [15, 16]. The animal experiments for establishing the tissue panel were approved by the Ethical Committee for the Treatment of Laboratory Animals at Chugai Pharmaceutical Co., Ltd.

Preparation of paraffin sections from a tissue block of human colorectal adenoma region

Paraffin sections for immunofluorescence staining were prepared from a block of a human adenocarcinoma tissue that was obtained from a patient that required surgery. Paraffin tissue blocks had been prepared by the standard method after fixating in 10% neutral buffer formalin. A part of an adenoma region was subject for the

observation in the adenocarcinoma tissue. The surgically excised tissue was provided by a patient that had given informed consent, as approved by the ethical committee at Kyushu University.

Immunofluorescence staining

The optimal concentration of the primary antibody for each staining method was determined as the concentration which yields positive reaction in LGR5+CSCs and no positive reaction in LGR5-CSCs.

The labeled streptavidin biotin (LSAB) method with Alexa Fluor 488 (Life Technologies) was conducted by the standard method. Briefly, paraffin sections from the above-mentioned blocks and then incubated with an anti-LGR5 antibody (Chugai, 2U2E-2, 2.5 μg/ml). Then, the sections were incubated with a secondary antibody conjugated with biotin (Abcam, Cambridge, UK), and the reaction was visualized by Streptavidin-Alexa Fluor 488-biotinylated (Life Technologies, 5.0 μg/ml).

The Qdot method was performed as recommended by the manufacturer. After incubation with the primary antibody (2.5 μg/ml), the sections were incubated with a secondary antibody conjugated with biotin (Abcam), and the reaction was visualized by Streptavidin-Qdot 605 (Life Technologies, 1/50 dilution).

The tyramide method was performed as recommended by the manufacturer. After incubation with the primary antibody (1.0 μg/ml), the sections were incubated with a secondary antibody conjugated with polymer-horseradish peroxidase (HRP) (DAKO), and the reaction was visualized by Alexa Fluor 488-labeled tyramide (Life Technologies, 1/100 dilution).

Antigen retrieval in each method was conducted by heating with microwave (H2850: Energy Bean Sciences, CT, USA) in a target retrieval solution (pH 6, Dako, Glostrup, Denmark) at 98°C for 15 min. The same specimens were also stained with DAPI or Qnuclear Deep Red Stain (Life Technologies) for nuclear counterstaining.

Detection and analysis of LGR5-positive cells

The slides made from paraffin blocks mentioned above were read under a Nikon A1+ confocal microscope (Tokyo, Japan). For each staining method, the laser power and detector was adjusted to a level at which LGR5-CSCs did not show any positive staining. To measure the degree of increasing background on histopathological slides, the appropriate conditions were fixed using LGR5-CSCs, and then photographs were taken, adjusting only the laser power.

Counting and measuring the intensity profile of LGR5-positive cells

After taking the above-mentioned photographs of slides made from three kinds of cell blocks, at least a hundred cells were counted in each of the photographic prints, and positive and negative cells were distinguished. The intensity profile of a hundred cells for the Qdot and the tyramide were measured by Imaging Software NIS-Elements (Nikon, Tokyo, Japan).

III. Results and Discussion

With the Alexa-LSAB method, 3.7% of the cells showed a positive reaction in LGR5+CSCs, only a few cells were positive with LOVO, and there were no positive cells with HCT116 (Fig. 1A, B). Because the positive rate was low with the Alexa-LSAB method, we next considered the Qdot method with a high-brightness dye, and found that the number of positive cells was increased compared to the Alexa-LSAB method (Fig. 1B), and the brightness of a positive reaction was higher than in the Alexa-LSAB method. However, since the positive cell rate of LGR5+CSCs was still lower than expected (50.5%), the tyramide sensitization method was tested. With the tyramide method, all LGR5+CSCs were positive (Fig. 1A, B), the number of positive cells was also increased in LOVO, and there were no positive cells in HCT116.

The staining profile was also different with each method. With the Alexa-LSAB method, a positive reaction was observed as tiny dots, whereas with the Qdot method, brighter spots were found, and with the tyramide method a positive reaction was observed diffusely throughout the whole cytoplasm (Fig. 1C). When, the difference in signal intensity in LGR5+CSCs measured by Imaging Software NIS-Elements between the Qdot and the tyramide methods was compared, some of the LGR5+CSCs showed staining with high intensity in the Qdot method, but all cells showed staining with high intensity in the tyramide method (Fig. 1D). The advantage of the tyramide method of enhancement is that the large size of the reaction product [14] makes it effective in enhancing a positive reaction evenly in low or high expression cells; however, subcellular distribution becomes less distinct. On the other hand, it was known that the staining pattern in the Qdot method accurately reflects the subcellular distribution and its staining intensity is quantitatively reflect the number of molecules that are expressed [13, 21]. Based on this, since it was reported that LGR5 is a receptor which is located mainly in the cell membrane [6], it was considered that the Alexa-LSAB and Qdot methods accurately detected LGR5 expression in cell membrane. The difference in detection sensitivity and staining profile for each method was caused by different staining principles. Therefore, the enhancement method should be selected carefully according to the purpose of the study.

In order to apply the method for histopathological studies, we attempted to detect LGR5 expression in normal monkey intestine and a human colorectal adenoma region by the Qdot and the tyramide methods. In the normal monkey intestine, both methods could detect LGR5-positive cells in the crypt base columnar cells (CBCs) located between the Paneth cells in the crypt base (Fig. 2A). CBCs are known as LGR5 expressing cells in normal intestine [2–

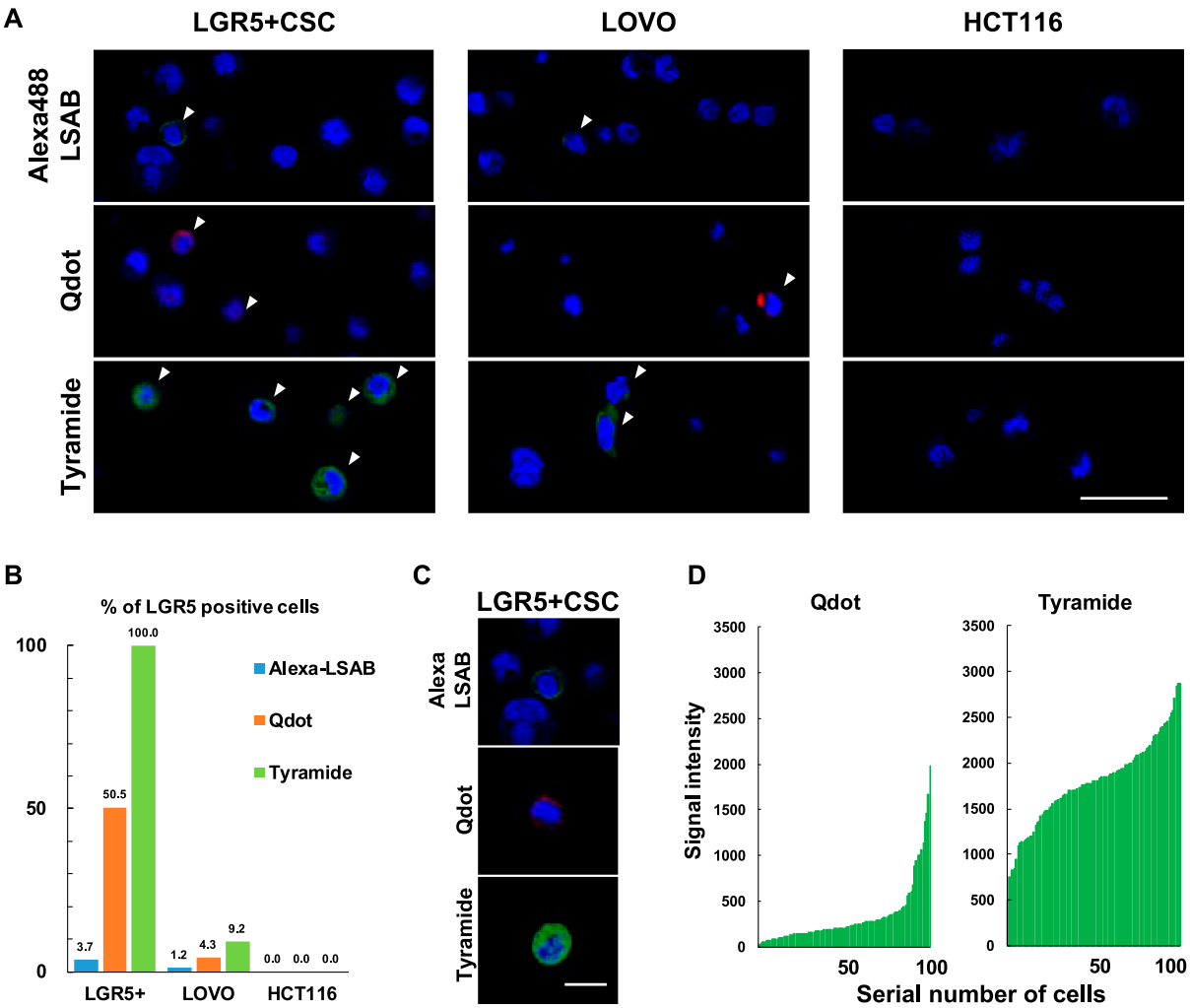


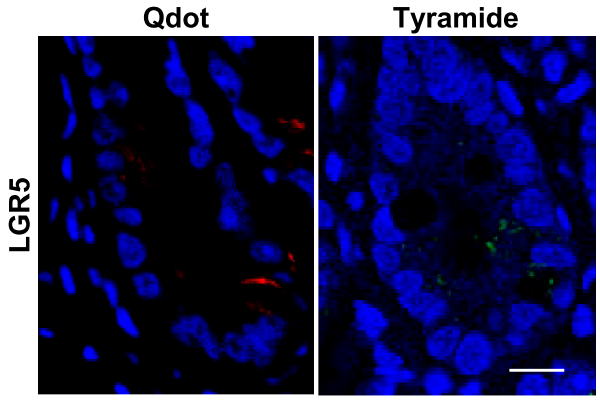
Fig. 1. Detection of LGR5 in cultured cells. (**A**) Photomicrographs of LGR5-positive cells detected by Alexa488-LSAB, the Qdot, and the tyramide methods in LGR5+CSC, LOVO, and HCT116. Bar=25 μm. (**B**) Positive rate of LGR5 by Alexa488-LSAB, the Qdot, and the tyramide methods in LGR5+CSC, LOVO, and HCT116. (**C**) Subcellular distribution of LGR5-positive reaction by Alexa488-LSAB, the Qdot, and the tyramide methods in LGR5+CSC. Bar=10 μm. (**D**) Bar chart of individual values for intensity profile in ascending order. Intensity profile of a hundred LGR5+CSCs with the Qdot and the tyramide methods measured by Imaging Software NIS-Elements.

4]. Both fluorescence staining methods enabled LGR5 to be detected on tissue samples, but there was a difference in their background staining patterns. Specific staining could be detected at a wide range of laser power levels with the Qdot method, and it was easy to distinguish specific staining from background staining. In contrast, the tyramide method had a narrow range of laser power levels at which specific staining and non-specific staining could be differentiated (Fig. 2B). In the human colorectal adenoma region, a positive reaction was observed in the basal area of crypt-like structures with the Qdot method (Fig. 2C) and the localization of LGR5-positive cells was similar to a previous report by Baker *et al.* [1]. The low background staining makes it easy to detect a positive reaction in the Qdot method, but high background staining makes it difficult to detect an appropriate positive reaction in the tyramide method, even when the level of laser power was tightly controlled.

The range of low expression cells that could be detected with the tyramide method was greater than the Qdot method, because the tyramide method is highly sensitive to low levels of antigen expression. However, it is critical to control the background staining when using the tyramide method, and the process of tissue preparation affects the preservation of antigens and the background staining. Thus we believe that the tyramide method is useful for samples collected under controlled conditions, such as xenograft tissues or tissues from experimental animals, and we previously used the method to identify colon cancer stem cells [8]. On the other hand, in line with the results in this study, a number of reports show that the Qdot method has a high S/N ratio [21]. Because clinical sampling is usually conducted under varying conditions, such as different fixation times, we recommend the Qdot method for clinical samples.

Current reports demonstrating the presence and nature of LGR5-positive cancer stem cells strongly suggest the

A



B

Relationship between laser power and positive reaction in Qdot

	Laser power				
Tissue	1.7	2.1	2.5	2.9	4.1
CBC	-	+	+	+	+
Other cells & tissue elements	-	-	-	-	+

Relationship between laser power and positive reaction in Tyramide

	Laser power			
Tissue	0.1	0.3	0.5	0.7
CBC	-	+	+	+
Other cells & tissue elements	-	-	+	+

C

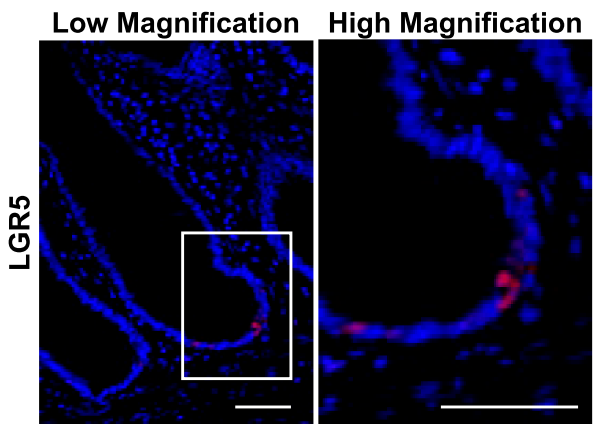


Fig. 2. Detection of LGR5 in tissues. (**A**) Photomicrographs of CBCs that have a positive reaction to LGR5 in the Qdot and the tyramide methods in the intestine of a normal cynomolgus monkey. Bar=10 μm. (**B**) Relationship between laser power and positive reaction in the Qdot and the tyramide methods in the intestine of a normal cynomolgus monkey. CBC in this figure means crypt base columnar cells. (**C**) Photomicrographs of LGR5-positive cells in human colorectal adenoma by the Qdot method. Bar=50 μm.

important role of LGR5-positive cancer stem cells in the development, progression, metastasis, and recurrence of cancer [20, 23]. To gain more insights into the pathophysiological roles of LGR5-positive cells and be able to develop therapeutic approaches targeting cancer stem cells, further fine analysis of the distribution and the fate of LGR5-positive cancer stem cells in human cancer tissues is necessary, and the methods evaluated in this study are useful for this purpose.

In conclusion, to detect LGR5 on tissue slides, it was considered important to select the staining method according to the purpose of the study. The tyramide method is superior to the Qdot method for intensifying low expression protein, while the Qdot method is superior to the tyramide method for identifying the subcellular localization of the target protein and for controlling the background staining in tissue samples.

IV. Declaration of Conflicting Interests

We have no conflicts of interest to declare.

V. Acknowledgments

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