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In vivo imaging of Lgr5-positive cell populations using confocal laser endomicroscopy during early colon tumorigenesis

Jin Woo Choi^{1,2,*}, Jun Ki Kim^{3,*}, Myunghwan Choi^{1,4}, Yi Rang Kim⁴, and Seok Hyun Yun^{1,**}

¹Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

²Department of Pharmacology and Institute of Dental Research, School of Dentistry, Wonkwang University, Iksan, Chonbuk, 570-749, Korea

³Biomedical Engineering R&D Center, Asan Institute for Life Sciences, Asan Medical Center and University of Ulsan College of Medicine, Seoul 138-736, Korea

⁴Department of Oncology, Asan Medical Center, University of Ulsan College of Medicine, Seoul 138-736, Korea

Abstract

Background and study aims—A diagnostic molecular marker for pre-neoplastic lesions, particularly before polyposis, is still lacking. Lgr5 has been broadly accepted as a marker for intestinal cancer stem cells. Monitoring Lgr5-positive cells is expected to provide a useful tool for early diagnosis of premalignant lesions before polyp formation.

Methods—*In vivo* molecular imaging was performed to examine colon tumorigenesis in *Lgr5-eGFP* mice treated with azoxymethane (AOM) and dextran sodium sulfate (DSS). eGFP⁺ regions in the descending colon were longitudinally monitored using side-view confocal endomicroscopy. Based on the eGFP signal intensity on the luminal surface, polyps were classified into two groups: Lgr5-high and Lgr5-low. White light colonoscopy was used to monitor polyp formation.

Results—About 75% of the polyps originated from foci containing eGFP⁺ Lgr5-positive cells, whereas 25% polyps emerge from Lgr5-negative foci. Among eGFP⁺ foci, Lgr5-high foci grew faster than Lgr5-low foci.

Conclusions—Polyps developed at Lgr5⁺ regions. Luminal Lgr5 expression was correlated with the growth rate of early-stage adenomas. Lgr5 is a promising molecular marker for early diagnosis of colon tumors.

Introduction

Colorectal cancer (CRC) is preceded by a pre-invasive (adenoma) state that lasts for several years.[1] Early detection and resection of adenomas are important to prevent colon cancer. Current endoscopic procedures using conventional endoscopes have several drawbacks. Colon tumors expressed flat or mildly elevated focal lesions are hard to detect.[2]

**Corresponding author: Seok Hyun Yun, syun@hms.harvard.edu.

* Authors contributed equally to this work.

Discriminating tumors from gross surrounding inflammatory lesions is difficult in cases with underlying inflammatory bowel disease (IBD), such as ulcerative colitis and Crohn's disease.[3] To overcome these problems, novel endoscopic techniques, including narrow-band imaging endoscopy [4,5], autofluorescence endoscopy [4,5], and confocal laser endomicroscopy (CLE) [6,7] have been introduced to improve clinical precision of cancer detection. Fluorescence imaging targeting specific molecular markers for pre-cancerous lesions can greatly improve early detection, but reliable biomarkers are currently lacking.

Stem-cell markers are promising candidates. Lgr5 is one of the most reliable intestinal stem-cell markers.[8] Lgr5 positive cells predominantly populate adenomas. Lgr5⁺ cells were found within tumors in an *Apc*-knockout mouse model and were shown to promote the growth of adenomas in the small intestine.[9] These results suggest that Lgr5 may define intestinal cancer stem cells as well as normal stem cells.

Here, we report *in vivo* observation of Lgr5⁺ cells in a mouse model of chemically induced, early tumorigenesis using a recently developed side-view CLE,[10] which enabled cellular imaging of the murine gastrointestinal tract over a wide area.

Methods

Tumorigenesis

Lgr5-EGFP-IRES-creERT2 knock-in transgenic mice (Lgr5-eGFP mice) were purchased from Jackson Laboratories (B6.129P2-*Lgr5^{tm1}(cre/ERT2)Cle/J*). Lgr5-eGFP mice (8 weeks) were injected intraperitoneally with 10 mg/kg azoxymethane (AOM, Sigma). One week later, the mice were given 3% dextran sulfate sodium (DSS, MP Biochemicals) in drinking water for 5 days followed by normal drinking water afterwards. The Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital approved the study.

Confocal endomicroscopy

A side-view optical probe (diameter: 1.3 mm, length: 5 cm) was inserted into the descending colon of mice anesthetized by intraperitoneal administration of ketamine/xylazine (90 mg/9 mg per kg body weight) (Fig. 1). eGFP was visualized by 491 nm excitation and 502–537 nm fluorescence detection. For vasculature imaging, rhodamin dextran conjugates (5 µg/µl, 2,000,000 MW, Invitrogen) were injected intravenously and imaged by 532 nm excitation and 562–596 nm detection. The coordinates (angle, distance from the anus) of each tumor were recorded to help identify the same foci in the following imaging session.

White-light colonoscopy

A white-light telescopic endoscope (outer diameter: 1.9 mm, ColoView, Karl Storz) using Xe lamp (XENON nova 175, Karl Storz) was used to monitor polyps at the coordinates recorded in confocal imaging. The tumor size was estimated from 3-chip video images (width×height×2), where the distance-dependent magnification was calibrated by imaging a ruler at the same distance as the tumor.

Cleared colon tissue imaging

Dissected colon tissues were cleared by using benzyl alcohol/benzyl benzoate solution (BABB) to increase the transparency for three-dimensional imaging of the crypt.[11]

Results

Migration of Lgr5⁺ cells to the colonic luminal surface after AOM treatment

Colon tumorigenesis was induced by a single injection of AOM or administration of AOM–DSS in Lgr5–eGFP mice. We then examined eGFP expression in the isolated colon segments using fluorescence microscopy. Under normal conditions, eGFP⁺ regions were confined in the crypt bottom and not observed on the luminal colon surfaces in eGFP–Lgr5 mice (Fig. 2A). eGFP⁺ foci emerged on the luminal surface 3 days after AOM treatment and were apparently confined to hexagonal vascular formations (Fig. 2A). To verify how eGFP signal moved to the luminal surfaces, we imaged Lgr5⁺ cells along the luminal transverse sections. Under normal conditions, Lgr5⁺ cells were restricted deep within the crypts (Fig. 2B). Upon AOM treatment, Lgr5⁺ cells formed clusters of 5–10 cells at luminal foci (Fig. 2B). Taken together, Lgr5⁺ cells migrated to the luminal surfaces during AOM-induced tumorigenesis.

Imaging Lgr5⁺ foci and the associated vasculature *in vivo*

Single AOM injection can induce microadenoma development accompanying angiogenesis. [12] *In vivo* confocal endomicroscopy revealed the progressive growth of Lgr5⁺ foci and angiogenic vessels on the luminal colonic surfaces over time (Fig. 3A). The area of eGFP⁺ region increased exponentially over time (Fig. 3B). Vasculature changed from regular hexagonal lattices to irregularly formed networks over time after AOM treatment. Neovascular patterns overlapped with high Lgr5⁺ cell populations are readily observed 4 weeks after AOM treatment (Fig. 3C).

Emergence of polyps at Lgr5⁺ foci

To cause polyposis, AOM treatment was followed by 3% DSS administration at 1 week intervals. Four weeks after the AOM–DSS treatment, a total of nineteen eGFP⁺ regions were observed in 7 mice by *in vivo* CLE and white-light colonoscopy (Fig. 4A). Only one small polyp was observed at week 4, but at week 10, 13 polyps emerged from the 19 eGFP⁺ sites marked at week 4 (Fig. 4B). We found 5 additional macroadenomas in these mice, but these polyps had emerged from eGFP-negative sites at week 8 to 10 (Fig. 4C). eGFP⁺ regions were detected typically 2–4 weeks before the appearance of macroscopic polyps that can be observed by white-light colonoscopy. This result indicates the potential of Lgr5 as an early diagnostic marker before polyposis.

Determination of tumor growth rate by Lgr5 expression level

Among the 18 tumors observed at week 10, 13 tumors had shown eGFP signals on the luminal surfaces at week 4. We further classified them into two groups: Lgr5-high (n=7) and Lgr5-low (n=6), based retrospectively on their average eGFP fluorescence intensity at week 4 (Supplementary Fig. 1). The difference of Lgr5 expression was confirmed by

immunostaining and BABB clearing imaging with isolated polyps (Supplementary Fig. 2). From our longitudinal data obtained with white-light colonoscopy, we found that tumors derived from Lgr5-high foci grew faster and larger than those originated from Lgr5-low foci (Fig. 5A and B). Histopathology at week 12 indicated a significant size difference between two representative tumors (Fig. 5C).

Discussion

Lgr5 is the most reliable marker for intestinal cancer stem cells as well as normal stem cells. In a previous study, Lgr5 was reported to contribute to non-inflammatory cancers.[9] In this work, AOM and DSS were used to mimic inflammation-associated tumorigenesis.[13] In early stages of tumorigenesis before polyposis, Lgr5⁺ cells that emerge on the colonic luminal surfaces can be readily detectable with help of appropriate fluorescent probes by CLE. According to our data, Lgr5 expression within tumors is correlated with the growth rate of early-stage adenomas in the inflammatory murine models. Thus, Lgr5⁺ cells may be used as an early diagnostic marker in both non-inflammatory and inflammation-associated colon tumors.

Through combination of Lgr5 and CLE, we showed the potential to predict the growth of colonic tumorigenesis foci much earlier than they are visible by conventional white-light colonoscopy. Although we utilized GFP-transgenic mice to monitor the role of Lgr5⁺ cells in this research, fluorescence-labeled antibody against Lgr5 can be clinically used to mark Lgr5⁺ cells in the near future. Subsequently, these likely tumor sites can be removed easily at initial stages without using high-risk endoscopic procedures such as endoscopic mucosal resection or endoscopic submucosal dissection. Further, CLE for visualization of Lgr5⁺ cells may become a predictive tool for prognosis after surgery. As disease relapse can be anticipated by measuring Lgr5 expression levels, tumor regrowth after surgery may be monitored by observing the extent of Lgr5⁺ sites on luminal surfaces. Furthermore, Lgr5⁺ cells may be appropriate targets for preventive therapies. In summary, the CLE technique complemented with detection of Lgr5⁺ cells may improve colon cancer prevention and advance therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. *In vivo* colonoscopy

(A) Schematic of the experimental setup for endomicroscopy in the descending colon of an anesthetized mouse. (B) Illustration of side-view endomicroscopy.

Fig. 2. Appearance of Lgr5-eGFP-positive sites

(A) Fluorescence images of a representative Lgr5-eGFP-positive region in the colonic luminal surface of an isolated murine colon. Green, eGFP signal; red, rhodamine-dextran in the vasculature. Scale bar, 100 μ m. (B) Two-photon images of colonic tissues isolated from eGFP-Lgr5 mice without (left) and 7 days after AOM treatment (right) and cleared by BABB. Blue arrows indicate Lgr5⁺ eGFP⁺ cells in the bottom of the crypts, whereas red arrows indicate Lgr5⁺ eGFP⁺ cells found near the apex of the crypts. Second harmonic generation signal (blue) from the collagen fibers delineate the crypt structure.

Fig. 3. Expansion of Lgr5-eGFP⁺ sites during tumorigenesis

(A) A representative Lgr5-eGFP⁺ region was monitored over 8 weeks following the last DSS treatment. Scale bar, 200 μ m. (B) The time-lapse change of the area with green eGFP signal in the region of interest (500 μ m $\times$ 500 μ m) of the mouse. (C) The area of red fluorescence from rhodamine dextran injected into blood vessels in the same region of interest. **, $p < 0.01$; ns, no significance.

Fig. 4. Macoscopic adenomas arise from eGFP⁺ regions

(A) Longitudinal measurement of polyps originating from two eGFP⁺ regions. Upper panel, 240° at 510–530 from the anus; lower panel, 240° at 400–425 from the anus. Images at the top row were obtained by fluorescence endomicroscopy; bottom rows were taken using white-light colonoscopy. Scale bars, 100 μm for confocal images. (B) Occurrence of polyps at Lgr5-eGFP-high regions observed over 10 weeks. (C) Comparison of the number of polyps emerging from eGFP⁺ vs. eGFP-negative regions.

Fig. 5. Lgr5-high tumors grow faster than Lgr5-low tumors

(A) Longitudinal imaging of Lgr5-high and Lgr5-low polyps 8–12 weeks after AOM–DSS treatment. Scale bars, 100 μ m for confocal images. (B) Tumor volumes measured over time for Lgr5-high (green, n=7) and Lgr5-low tumors (purple, n=6). (C) Representative Lgr5-low and Lgr5-high tumors at week 12. Scale bar, 1 mm. **, $p < 0.01$; ns, no significance.