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Photodynamic Diagnosis of Colitis-associated Dysplasia in a Mouse Model After Oral Administration of 5-Aminolevulinic Acid

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Abstract. *Background: Detection of dysplastic lesions in mouse colonic tissue was investigated by accumulation of photosensitizer protoporphyrin IX (PpIX) induced by oral administration of 5-aminolevulinic acid (5-ALA), a precursor of PplX. Materials and Methods: Inflammatory oncogenesis was induced in C57BL/6J-Apc^{Min} (Apc$^{Min/+}$) mouse by oral administration of dextran sodium sulfate (DSS). After oral administration of 5-ALA, the colonic tissue was observed by autofluorescent stereoscopy and histopathological examination. The localization of fluorescence signals in the colonic tissue was determined with a mobile ultraviolet Xe lamp light. Results: Several small polypoid lesions were found in the mucosal layer of DSS-treated Apc$^{Min/+}$ mice. Strong reddish ring-shaped fluorescence signals of PpIX, at 635 nm measured by a spectrum analyzer, were observed on the mucosal surface of all protruding lesions that were confirmed to be histopathologically dysplastic . Conclusion: Photodynamic diagnosis with 5-ALA was useful in detecting dysplastic lesions in the colonic mucosa in a mouse model.*

Ulcerative colitis (UC) is a chronic inflammatory disorder of the large intestine. It has been associated with various complications, and colitic cancer is one of the most important complications in patients with long-standing UC. Recently, the incidence of UC has been increasing. Thus, early and accurate detection of dysplastic lesions has become an important issue in clinical management of patients with long-standing and extensive UC (1). Dysplasia of pre-cancerous lesions is a predictor of colitic cancer; thus, the detection of both colitic cancer and dysplasia (colitis-associated cancer/dysplasia, CC/D) in colonic biopsies is significant for UC surveillance (1). Some prospective studies showed that chromoendoscopy using indigo carmine and methylene blue combined with target biopsy was more successful than conventional colonoscopy in identifying dysplastic lesions (2, 3). Therefore, such endoscopic procedures have been proposed for a more efficient detection of CC/D; however, despite the use of this procedure, it is difficult to accurately diagnose CC/D, because of the characteristic shapes of the lesions. Recently, new innovative endoscopic procedures, such as narrow-band imaging (NBI) endoscopy and autofluorescence endoscopy (AFE), have been utilized for the detection of CC/D; however, their effectiveness remains unclear (4). Photodynamic diagnosis is one such innovation that has been used clinically to detect the extent of neoplasms, especially in neurosurgery (5) and urology (6).

For photodynamic diagnosis of CC/D in UC, 5-aminolevulinic acid (5-ALA), which itself does not emit fluorescence signals, is utilized. However, in the tumor cells, 5-ALA is converted to the sensitizer protoporphyrin IX (PpIX), that selectively accumulates in neoplastic tissue, facilitating its detection (7). Therefore, photodynamic diagnosis using 5-ALA may be a promising technique for UC surveillance in humans. However, very few studies have reported its use with 5-ALA for UC surveillance (7-9), and the results of its clinical utility and significance are unclear (9). This is because the relationship between neoplastic lesions and fluorescence signal patterns remains unclear (7, 9).

Knowledge of fluorescence signal localization in an animal model of UC will help us gain some insight into the fluorescence signal patterns of CC/D in human UC. Dextran sodium sulfate (DSS) is known to strongly induce intestinal carcinogenesis along with strong oxidative and nitrosative

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stress, since it induces inflammation, in C57BL/6J-*Apc*^{Min} (*Apc*$^{Min/+}$) mice, a model for familial adenomatous polyposis (11-17). This mouse model can be used in the investigation of the pathogenesis of inflammatory bowel disease because the β-catenin signaling pathway may be involved in the carcinogenesis of UC (12, 17-20).

In this study, we determined the precise localization of PpIX in the large intestine of *Apc*$^{Min/+}$ mice by reading fluorescence signals following oral administration of 5-ALA, in order to assess the usability of photodynamic diagnosis using 5-ALA in detecting neoplastic lesions in UC.

Materials and Methods

Reagents. 5-ALA (pure powder; Cosmo Bio, Tokyo, Japan) was dissolved in 200 ml of distilled water. DSS was purchased from MP Biochemicals, LLC (Aurora, OH, USA) and dissolved in distilled water to obtain a concentration of 2%.

Animal model. Twenty male *Apc*$^{Min/+}$ mice (15) (14 test mice and 6 control mice) aged 8-12 weeks were a gift from Dr. Sadamu Homma, the professor of Institite of DNA Medicine, Jikei University School of Medicine. C57BL/6J mice and *Apc*$^{Min-/+}$ mice were purchased from Nihon SLC Co. Ltd. (Hamamatsu, Japan) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively. All experimental procedures were performed in accordance with the Jikei University guidelines on animal welfare.

Another control group of three wild-type mice was formed. They were given 2% DSS-containing drinking water for the first seven days; no further treatment was administered for the next four weeks in order to allow for the development of inflammatory oncogenesis (12-21). In the fifth week, the mice were euthanized three hours after administration of oral 100 mg/kg 5-ALA, according to the dose used by Loh *et al*. (22). The entire colon containing the induced colonic dysplastic lesions was removed, cut longitudinally, and opened. *Apc*$^{Min/+}$ mice without administration of 5-ALA were utilized as the negative control. Another control group comprised of wild-type mice that received the same dose of 5-ALA as test mice. Both controls were examined macroscopically and microscopically.

Histopathological studies. For light microscopic observation, the resected colon was fixed in 10% formalin, and paraffin-embedded thin sections of the resected colon were deparaffinized through a graded series of ethanol and xylene. These sections were counterstained using hematoxylin-eosin and mounted. The dysplastic lesions were then examined. The colonic tissue specimens containing the histopathologically-diagnosed dysplastic lesions were treated the same way as the colon. Each slide was examined histopathologically for inflammation, dysplasia, and carcinoma. In addition, immunohistochemical analyses for Ki-67 were performed to compare dysplasia, carcinoma, and surrounding normal tissue. In brief, a monoclonal antibody to Ki-67 (ab15580, Abcam®, Cambridge, UK) was utilized as the primary antibody using the ABC method (Vectastain® ABC-kit; Vector Laboratories, Burlingame, CA, USA). In addition, inflammation was diagnosed histopathologically.

Fluorescence studies. To detect fluorescence signals, a mobile ultraviolet Xe lamp light source was used, and the wavelength of the fluorescence signal was confirmed by using an analyzer (VLD-M1/ver. 3.0SP; M&M Co., Tokyo, Japan).

To prevent photobleaching of the fluorescence signals, the large intestine was first examined from the colonic serosa side macroscopically, immediately after euthanasia. The entire colon was removed, cut longitudinally, and investigated for fluorescence signals by using a mobile ultraviolet Xe lamp light. Macroscopic localization of the strong fluorescence signals was confirmed by excitation using blue light at 405 nm, detected at 635 nm, filtering the yellow light (22). Fluorescence signals were compared with the macroscopic observations to identify the correlation between strong fluorescence signals and dysplastic lesions. The strong signals at 635 nm, which had a fluorescence spectrum typical of PpIX, were also monitored using a spectrum analyzer VLD-M1/ver. 3.0SP.

Next, the colon along with its fluorescence signals was fixed in 4% paraformaldehyde containing phosphate-buffered saline (PBS), replaced with 20% sucrose and 10% glycerol in PBS, cut into 100-μm thick sections, mounted, and examined with both a microscope (SMZ 1000; Nikon, Tokyo, Japan) and an autofluorescence stereomicroscope (LSM 510; Carl Zeiss Japan, Tokyo, Japan) to confirm the histological localizations of fluorescence signals and their microscopic patterns in dysplastic lesions. All fluorescence examinations were performed in a dark room to prevent photobleaching (23-26).

Ethical considerations. This study was approved by the institutional Animal Care and Use Committee, as No. 23-014, at Jikei University, Tokyo, Japan.

Results

Characterization of Apc$^{Min/+}$ *mice treated with DSS*. Flat protruding lesions in the colon of DSS-treated *Apc*$^{Min/+}$ mice were seen macroscopically. These lesions were irregular, with a max dimension of approximately 2-5 mm, and were found to be surrounded by normal colonic mucosa (Figure 1). No massive invasion or metastasis to other organs was observed (Figures 1 and 2). No protruding lesions were found in the control mice.

Mild inflammation was observed in all colonic mucosa obtained from the *Apc*$^{Min/+}$ mice treated with DSS (Figure 2). On the other hand, several protruding lesions were observed to be confined to the mucosal layer of the DSS-treated mice (Figure 2). These lesions exhibited cellular and structural atypia, *i.e*., dysplastic changes, that ranged from low-to-high grade, according to the modified histopathology. Moreover, immunohistochemistry showed that the protruding lesions of the colon were diffusely-positive for Ki-67 (Figure 3), suggestive of proliferation of tumor cells. This finding was in line with that of the histopathological differentiation of low-to-high grade dysplasia.

Ring-shaped fluorescence signals at the margin of the protruding lesions. Macroscopic observation of the serosal side of the colon showed ring-shaped fluorescence signals arising from the mucosal side (Figure 4). Following the use of blue light at 405 nm for excitation, intense ring-like

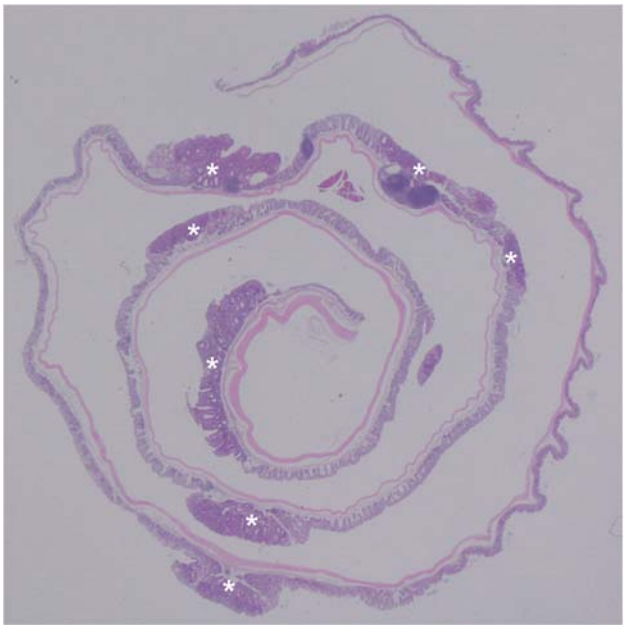

Figure 1. *Macroscopic findings of colonic polypoid lesions. Several polypoid lesions (asterisk) were found in the large intestine of $Apc^{Min/+}$ mice after administration of dextran sodium sulfate.*

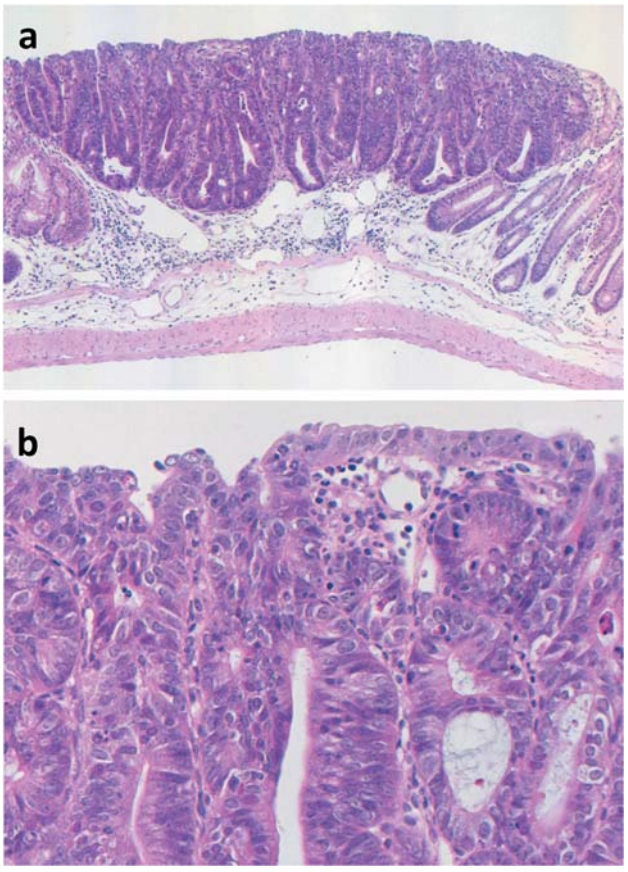

Figure 2. *Light microscopic findings of the colonic polypoid lesions. The colonic neoplasia is observed in the mucosal layer above the muscularis mucosa of the large intestine under low magnification (a). Under high magnification (b), the colonic neoplasia is seen to be a tubular adenoma. It was categorized as low-to-high grade dysplasia (a, ×100, b, ×400; hematoxylin-eosin staining).*

reddish fluorescence signals emitted by PpIX were observed, only along the margins of the protruding dysplastic lesions, but not outside or on the border of the lesions (Figure 5). The fluorescence spectra of the protruding tissue regions were measured with a spectrum analyzer that used an ultraviolet diode laser-induced fluorescence measurement system (Figure 6). The edges of the protruding lesions with strong fluorescent signals were observed at a wavelength of 635 nm, which is the fluorescence spectrum typical of PpIX. In contrast, the area adjacent to the protruding lesions produced a peak at around 500 nm, displaying autofluorescence, known to be intense in normal tissues and weak in tumor tissues. Moreover, the central area of the protruding lesions exhibited intermediate fluorescence signals, with an intensity measuring somewhere between that of the surrounding area and the edges of the lesions (Figure 6). The protruding lesions thus had fluorescence signals at their edges and relatively weak signals towards their center. A wavelength of approximately 400 nm was emitted from the ultraviolet diode laser itself. Macroscopically, the intensity of fluorescence signals from PpIX was clear and intense red towards the edges, and weak towards the center of the lesion (Figure 6). These fluorescence signals disappear due to bleaching within a short time period, and hence, care should be taken to observe them as soon as possible. No fluorescence signals were observed in the control mice.

Pharmacokinetics of PpIX in the protruding dysplastic lesions. To confirm the pharmacokinetics of PpIX in the protruding dysplastic lesions, the pattern of fluorescence localization from the serosal layer to the mucosal layer of the colon was investigated using a stereomicroscope with fluorescence. The intense reddish fluorescence signals of PpIX were observed towards the mucosal side of dysplastic lesions, histopathologically corresponding to the dysplastic regions that were not located deep below the muscularis mucosa layer (Figure 7). Moreover, reddish signals of PpIX were observed only in the protruding lesions. As the dysplastic tissues exhibited such strong fluorescence signals at the margins, strong fluorescence signals of protruding dysplastic lesions were also observed macroscopically on the serosal side of the colon but only along the margins of the lesions (Figure 4).

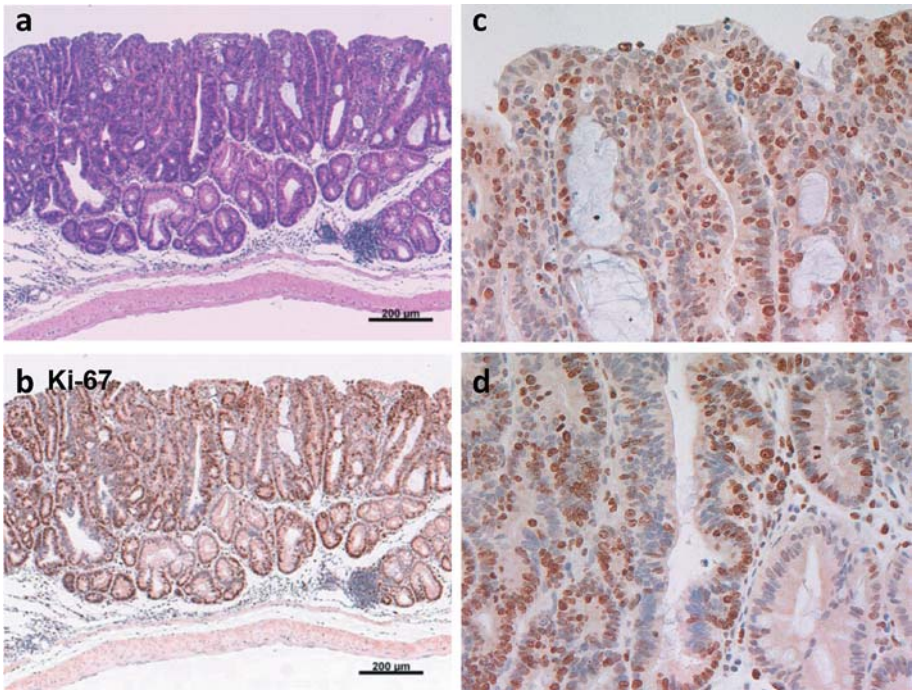

Figure 3. *Immunohistochemical findings of dysplasia. a: Light micrograph of dysplasia with hematoxylin-eosin staining under low magnification (a, ×100; hematoxylin-eosin staining). b: Immunohistochemical findings of dysplasia in the large intestine. Dysplasia exhibited diffuse positive reactions to antibody to Ki-67 under low magnification (b, ×100; Ki-67 staining). Under high magnification (c, d), positive reactions to antibody against Ki-67 can be seen, diffusing from the surface (c) to the bottom (d) (c, ×400, d, ×400; Ki-67 staining).*

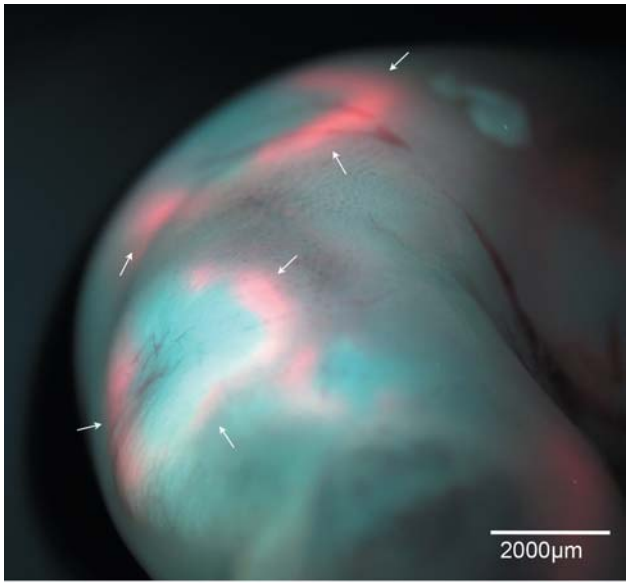

Figure 4. *Macroscopic findings obtained using a mobile ultraviolet Xe lamp. Macroscopic observation of the colonic serosa by using a mobile ultraviolet Xe lamp revealed strong reddish ring-shaped fluorescent signals (arrows).*

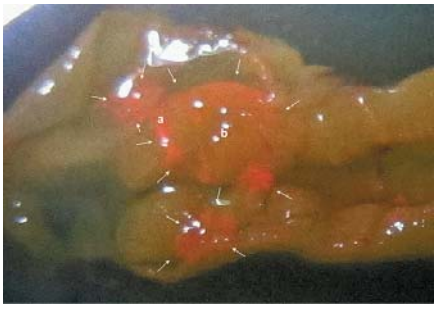

Figure 5. *Fluorescence study for the detection of neoplasia in the large intestine. Small protruding lesions (arrows), histopathologically-identified as dysplasia, exhibited strong reddish ring-shaped fluorescence signals in the margin (rounds), and relatively very weak fluorescence signals in the center.*

Discussion

CC/D is one of the most important complications in patients with long-standing UC. To detect CC/D at its earlier stages, some endoscopic procedures have been developed, such as chromoendoscopy, magnified endoscopy with NBI (27), and

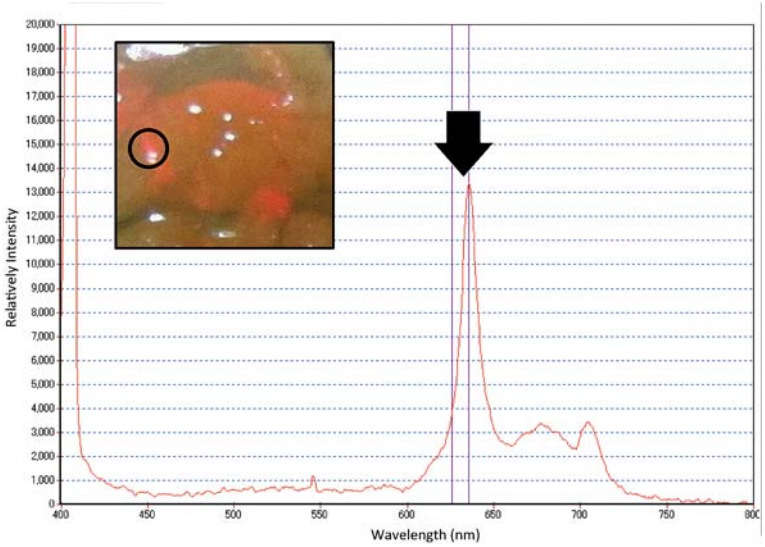

Figure 6. *Wavelength graph of the spectrum analyzer. Wavelength of the reddish ring-shaped fluorescence signals (black circle) was checked using a spectrum analyzer. A wavelength of 635 nm that is characteristic of PpIX metabolized from 5-ALA was clearly observed (arrows). Low broad-spectrum wavelengths with a peak at around 500 nm were observed as autofluorescence of normal tissues. A wavelength of 400 nm was derived from the ultraviolet diode laser itself. The relatively weak fluorescence signals with wavelengths above 635 nm may have arisen from the central area of the neoplasia.*

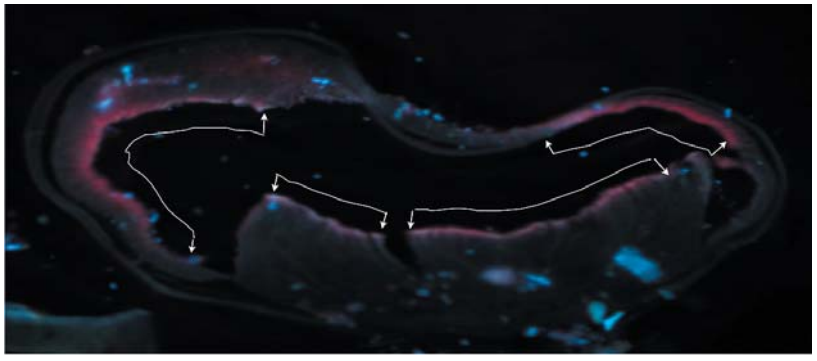

Figure 7. *Autofluorescence stereomicroscopic findings. The mouse colonic tissue was examined by autofluorescence stereomicroscopy to avoid bleaching. The reddish fluorescence signals (arrows) were confirmed to be emitted by the dysplastic lesions in the mucosal layer.*

AFE (28). Although these procedures have been evaluated for their clinical usefulness, their effectiveness in detecting CC/D remains controversial. Among them, AFE is considered to be one of the most effective, especially in cases of widespread flat elevated lesions, such as laterally-spreading tumors (29). However, UC sometimes presents with mucosal inflammation, and on AFE, the inflamed mucosa appears magenta compared to the non-inflamed green mucosa. Therefore, AFE alone is not enough for the detection of neoplastic lesions in cases of UC with mucosal inflammation. To resolve this problem, photodynamic diagnosis with 5-ALA has been introduced and evaluated (7-9). However, the endoscopic findings, such as localization of the fluorescence signals in CC/D, are still unclear (9). To understand the localization of the fluorescence signals of CC/D in human UC, it is very important to study the fluorescence signal patterns in animal models (19).

5-ALA is a natural amino acid biosynthesized in humans (30). Following systemic administration, 5-ALA in tumor cells is metabolized to PpIX, a porphyrin that serves as an endogenous photosensitizer (30). In addition, 5-ALA is water-soluble and can be administered locally, systemically, and orally. Although side-effects of 5-ALA such as nausea, photosensitivity reactions, and photodermatosis have been reported, their incidence was found to be only 0.1% (5-9, 30-31). Together with light or laser irradiation, 5-ALA is used

as a photosensitizer precursor in photodynamic diagnosis to identify tumor cells; this serves as a new technique for cancer diagnosis in the fields of neurosurgery (5), urology (6), and digestive surgery (31).

Although there are many animal models for UC, few can be employed to study the dysplasia-adenocarcinoma sequence. Tanaka *et al*. reported the development of dysplastic changes and colonic neoplasms, including adenocarcinomas, in the colon of $Apc^{Min/+}$ mice after oral administration of DSS (11). $Apc^{Min/+}$ mice display loss of heterozygosity of *Apc* and have no mutations in the *β-catenin* and *K-ras* genes (11, 12). This model is most suited to studying the dysplasia-adenocarcinoma sequence, because administration of DSS induces inflammation and strong oxidative/nitrosative stresses, which in turn promotes intestinal carcinogenesis (11, 12, 16, 17). Thus, we used $Apc^{Min/+}$ mice to observe the fluorescence signals of PpIX that was metabolized from orally-administered 5-ALA.

In $Apc^{Min/+}$ mice, dysplastic lesions that are very similar to CC/D lesions in humans were recognized as polypoid lesions in the large intestine. After oral administrering 5-ALA to $Apc^{Min/+}$ mice, strong reddish fluorescence signals were observed in the polypoid dysplastic lesions of the large intestine. These fluorescence signals seemed to be concentrated at the margins of the dysplastic lesions. However, the spectrum analyzer showed that the fluorescence signals were spread throughout the polypoid lesions, and that intense fluorescence signals, in particular, appeared as ring-like formations toward the margins of the lesions. These ring-like reddish fluorescence signals on the margins of tumor lesions are characteristic of CC/D. Previous studies using 5-ALA reported similar results with regard to the characteristic localization of the fluorescence signals in the lesion. For example, Stummer *et al*. reported the same pattern of fluorescence signals (intense signals at the margins) in their clinical experience, and stated that the central region without PpIX might contain necrotized tissue (5). Messmann *et al*. also reported results similar to ours, and stated that several factors, such as concentration of 5-ALA, status of mucosal inflammation, and method of administration of 5-ALA, could influence the intensity or distribution of fluorescence (7, 19). In the current study, no necrotic tissues were observed in the dysplastic lesions, and the status of mucosal inflammation was the same as that in non-polypoid lesions. However, the relationship between the intensity of fluorescence signals and the method of 5-ALA administration needs to be determined and examined.

The ring-like fluorescence signals emitted after 5-ALA administration are characteristic of dysplastic lesions and can, thus, be useful in detecting CC/D in humans. CC/D-related neoplastic changes may occur in flat or raised lesions in the colonic mucosa of humans. However, 95% of dysplastic foci occur in flat mucosa, most often in mucosa that appear non-suspicious macroscopically (32-33). Our macroscopic findings using 5-ALA are very helpful in identifying these lesions, owing to the characteristic signal localization pattern of CC/D in humans with UC.

As far as we are aware of, our study is the first to investigate macroscopically and histopathologically 5-ALA-induced fluorescence in an animal model of CC/D with the aim of improving surveillance in humans with long-standing UC. We conclude that fluorescence signals of dysplastic lesions are intense towards the margins and weaker towards the center, and that photodynamic diagnosis using 5-ALA is a promising method for detecting CC/D lesions in patients with long-standing UC.

Competing Interests

The Authors declare that they have no competing interests.

Acknowledgements

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