의학박사 학위논문

소화기 전암병변의 검출을 위한 목시플록사신과 프로플라빈 이중 형광 영상의 유효성 평가: 전향적 연구

Feasibility of moxifloxacin and proflavine dual fluorescence imaging for detecting gastrointestinal premalignant lesions: A prospective study

울 산 대 학 교 대 학 원

의 학 과

남 광 우

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이 논문을 의학박사 학위 논문으로 제출함

2022 년　2 월

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이번에 박사학위 논문을 무사히 마무리할 수 있도록
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중개연구의 흥미로운 세계를 경험할 수 있는 기회를 주시고
세심하게 프로젝트를 이끌어주신 명승재 지도교수님께
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끊임없는 열정과 참신한 아이디어로 연구를 이끌어주신
김기현 교수님 그동안 수고 많으셨고 감사드립니다.
원활한 연구 진행을 위해 검체 촬영 장비를 싣고 먼 거리를 이동하면서
마지막 데이터 정리 과정까지 고생 많으셨던
이승훈 박사님, 박노성 선생님 수고 많으셨고 고맙습니다.
바쁘신 와중에도 이번에 제자를 위해 학위논문 심사위원을 맡아주셔서
박사학위논문을 꼼꼼히 검토해 주시고 좋은 의견들을 제시해 주신
김도훈 교수님, 양동훈 교수님, 이정훈 교수님께 감사드립니다.

박사학위 연구 주제를 정하고 연구를 진행하고 논문을 정리하는
일련의 과정을 통해 연구의 시작부터 끝까지를 전체적으로 경험해보는
유익한 경험을 해 볼 수 있었습니다.

감사의 글 (2)

이번의 소중한 경험을 바탕으로 박사학위 연구 주제인
내시경 영상 분야에 계속 관심을 갖고 공부하면서
앞으로 후속 연구를 통해 관련 지식을 계속 발전시킬 수 있도록
꾸준히 노력해 보겠습니다.

끝으로 사랑하는 가족들에게 감사 말씀 드리고 싶습니다.
언제 어디서나 든든한 버팀목이 되어주시는
어머니와, 장인, 장모님 항상 고맙습니다.
또 지금까지 박사과정을 전폭적으로 지원해주느라 고생 많았던
사랑하는 아내와 소중한 아들 정말 고맙습니다.

국문요약

배경: 최근 내시경 영상 기술의 발달로 내시경 시술을 하면서 육안적인 진단이 가능하게 되었다. 고대비 고해상도 영상 기술은 병변을 실시간으로 민감하게 발견할 수 있게 한다. 본 연구에서는 목시플록사신과 프로플라빈을 이용한 새로운 이중 형광 영상을 소화기 전암성 및 조기암 병변에 적용하여 그 유용성을 조사하였다.

방법: 대장과 위의 전암성 및 조기암 병변이 확인된 환자들을 전향적으로 모집하였다. 병변을 생검겸자나 내시경 절제술로 절제한 후, 즉시 체외에서 목시플록사신과 프로플라빈을 도포하고 특수제작한 축방향 이동 광시야 형광 현미경으로 이중 형광 영상을 촬영하였다. 촬영 결과는 각각의 조직학적 검사 결과와 비교되었다.

결과: 여덟 명의 환자에서 열 개의 대장 병변이 (정상 1, 선종 8, 조기암 1) 네 명의 환자에서 여섯 개의 위 병변이 (정상 1, 선종 3, 조기암 2) 분석에 포함되었다. 목시플록사신과 프로플라빈을 이용한 이중 형광 영상은 자세한 세포의 형태, 배열, 구조를 보여주었다. 대장과 위의 정상 점막 검체에서는 정상적인 샘 구조와 세포 배열이 보존되어 있었다. 대장 정상 점막 조직에서는 배상세포가 확인되었다. 선종이나 조기암 조직에서는 불규칙한 샘 구조와 세포질 감소 소견이 관찰되고 핵의 모양이 길어져 있었다. 대장 병변에서는 배상세포가 잘 관찰되지 않았다. 이중 형광 영상간의 유사성 분석에서는 선종과 조기암에서는 정상 점막 조직에 비하여 상대적으로 높은 유사성을 보였다. 이중 형광 영상은 조직학적 검사 결과와 비교했을 때 대장 병변(82.3%)과 위

병변(86.0%)에서 좋은 정확도를 보여주었다.

결론: 목시플록사신과 프로플라빈을 이용한 고대비 고해상도 이중 형광 영상은 위장관 전암성 및 조기암 병변에서 자세한 조직학적 정보를 얻는 데에 유용하였다. 이중 형광 영상을 체내의 실시간 육안적 진단 방법으로 개발하기 위한 추가적인 연구가 필요하다.

중심 단어: 형광 영상; 위장관; 목시플록사신; 프로플라빈; 전암성 병변

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Introduction

Recent advances in gastrointestinal endoscopy have enabled the early diagnosis of neoplastic lesions in the gastrointestinal tract. Since most gastrointestinal neoplasms develop from premalignant lesions (dysplasia, metaplasia or adenoma) to cancer, early detection enables endoscopic resection and prevents surgical resection. To date, several high-contrast wide-field imaging methods, including chromoendoscopy, image-enhanced endoscopy (IEE), autofluorescence imaging (AFI), and magnification endoscopy, exist which aid in real-time visual diagnosis ("optical biopsy") [1] and to decrease missed lesions during gastrointestinal endoscopy [2,3]. Among them, IEE has shown promising data in the evaluation of gastrointestinal premalignant and early cancerous lesions, and thus is widely used in daily practice [4-6]. However, IEE is based on the structural and vascular changes of the mucosa, and in some previous studies, the polyp detection rate in colonoscopy with IEE was not superior to that of conventional white-light colonoscopy [7,8]. Thus, a more precise endoscopic imaging method is needed for daily practice.

High-resolution imaging methods, which can visualize glandular and cellular structures, enable real-time early detection of lesions. Endocytoscopy (EC) can visualize microstructures with high magnification using cell-labeling agents, such as methylene blue (nucleus), toluidine blue (nucleus), and crystal violet (cytoplasm) [9,10]. Confocal laser endomicroscopy (CLE) can visualize 3D cellular structures by using fluorescein as a contrast agent [11]; however, it has a small field of view (FOV) and slow speeds; thus, it would be more preferred for close cellular examination of detected lesions rather than detection of the lesion. High-resolution microendoscopy (HRME) can visualize cellular structures on the surface using proflavine as a cell nucleus-labeling agent. Proflavine hemisulfate, a disinfectant used for topical antiseptics, showed intrinsic fluorescence [1,12]

and has been used for cell nucleus labeling in low concentration (0.01%). HRME has a relatively large FOV and high speeds compared to CLE. HRME using proflavine showed effectiveness in differentiating neoplastic from non-neoplastic polyps [13,14].

Moxifloxacin, which is a fourth-generation fluoroquinolone, has been used as a cell-labeling agent for fluorescence imaging of eye, skin, bladder, and brain tissues of animal models [15-19]. Moxifloxacin highlighted glandular structure and arrangement with fluorescence enhancement compared to AFI [17,20]. In addition, goblet cells in the colonic crypt were well visualized with stronger fluorescence compared to epithelial cells [17,20,21]. Moxifloxacin is similar to proflavine in terms of intrinsic fluorescence and topical instillation for labeling with good tissue penetration [22-24]. Moxifloxacin and proflavine labeled the cell cytoplasm and nucleus, respectively. Therefore, they can be used in combination for the visualization of both cell cytoplasm and nucleus in fluorescence imaging.

Currently, there are no data on dual fluorescence imaging of moxifloxacin and proflavine in the human gastrointestinal tract. In this study, we investigated and compared the feasibility of dual fluorescence imaging in the gastrointestinal tract, including the normal mucosa and premalignant lesion tissues, with conventional histologic examination.

Patients and methods

Patients

Patients who underwent upper and lower gastrointestinal endoscopy for premalignant and early cancerous lesions in the colon and stomach were prospectively enrolled between January and August 2021. All enrolled patients agreed with the study protocol and provided informed consent for the endoscopic and imaging procedures. Patients who did not agree with the study protocol or had significant comorbidities that preclude endoscopic procedures were excluded. The study protocol was approved by the institutional review board of both the Asan Medical Center and Dankook University Hospital.

Endoscopic procedure

For colonic and gastric lesions, forceps biopsy or endoscopic resection (endoscopic mucosal resection or endoscopic submucosal dissection) was performed by a single gastrointestinal endoscopist. For the control, normal tissues in the colon and stomach were obtained by forceps biopsy. After collecting the specimens, the on-site dual fluorescence imaging using moxifloxacin and proflavine was performed. Then, the specimens were formalin-fixed and processed for the histological evaluation.

Ex vivo dual fluorescence imaging

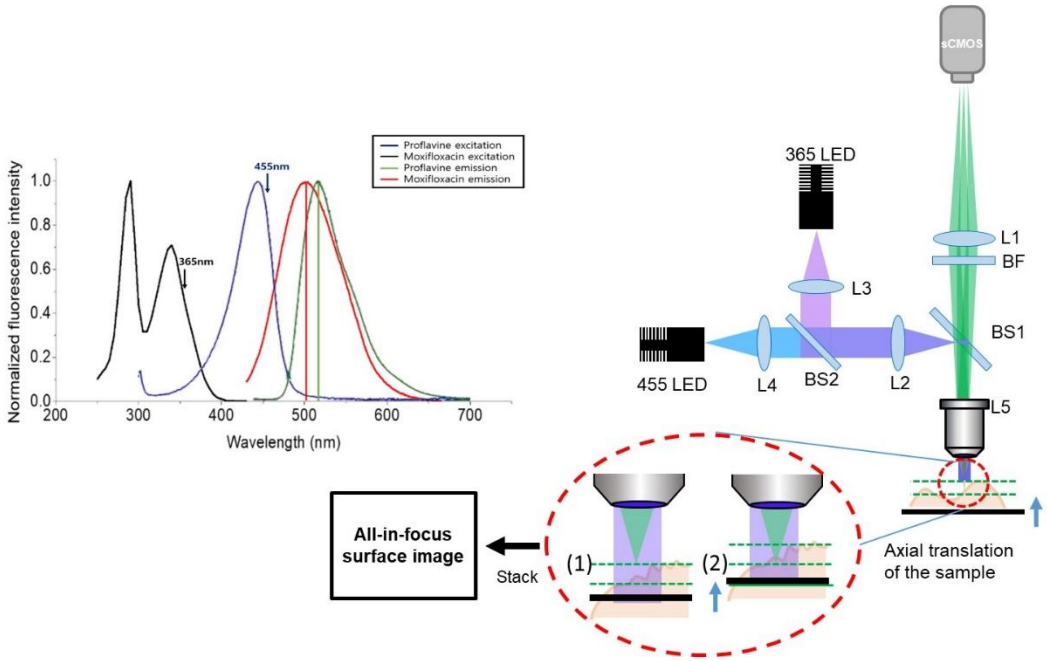
On-site dual fluorescence imaging was performed using the following steps. The specimens were washed with phosphate buffered saline (PBS) and labeled with moxifloxacin and proflavine sequentially via topical instillation. Moxifloxacin ophthalmic

solution (Vigamox®, Alcon) containing 12.4 mM of moxifloxacin hydrochloride was topically administered on the specimens and left for 5 min, followed by rinsing with PBS thrice [21]. The same procedure was followed for proflavine hemisulfate 0.01% solution. Dual fluorescence imaging was performed immediately after the topical labeling.

Dual axially-swept wide-field fluorescence microscopy

To visualize microstructures on the irregular surface of the specimens in focus, we developed axially-swept wide-field microscopy (1.2 mm FOV; 40 fps imaging speed; 1 mm depth-of-field) (Figure 1). The specimen was axially translated stepwise with a step size of 0.01 mm. Multiple acquired images were merged to generate all-in-focus images. Two light emitting diodes (LEDs) of 365 nm and 455 nm in wavelength were used for the excitation of moxifloxacin and proflavine, respectively. The two LEDs were alternately turned on and off with a triggering signal for fast sequential imaging. The emission spectra of both moxifloxacin and proflavine had peaks at approximately 500 nm. Emission light was collected by an sCMOS camera (Edge4.0, PCO, Germany) for image gneneration. Dual fluorescence images were generated by merging both moxifloxacin and proflavine images in different colormaps.

Figure 1. Schematic of dual axially-swept wide-field fluorescence microscopy.



L1: Acoromatic lens (f =200 mm), L2: Lens f = (100 mm), L3 and L4: Aspheric Condenser Lenses (f=20 mm), L5: Objective lens (20x, NA 0.4), BF: Emission filter (500 LP), BS1: Dichroic mirror (495 LP), BS2: Dichroic mirror (425 LP). Red ellipse describes the way of axial sweeping

Conventional and virtual histological examination using confocal fluorescence microscopy

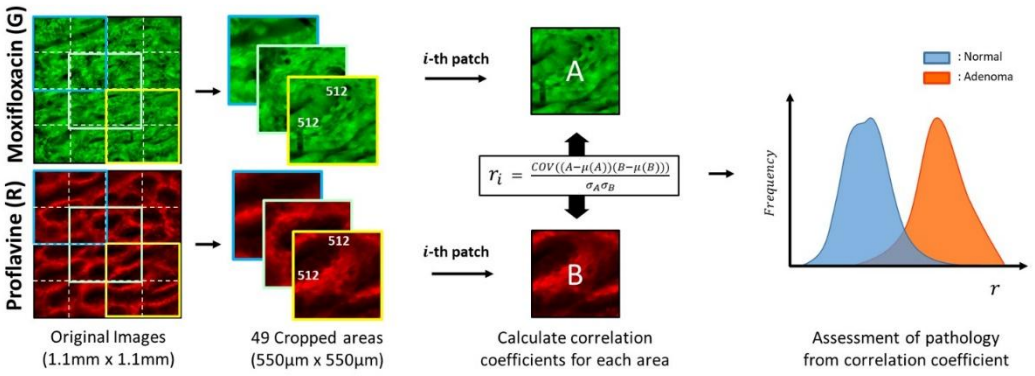
After dual fluorescence imaging in fresh condition, the specimens were fixed in 4% formaldehyde, embedded in paraffin, and sliced with a microtome. Sliced tissue slides were deparaffinized with xylene, rehydrated with alcohol, washed with distilled water, and stained with hematoxylin & eosin (H&E). The dual fluorescence images were compared with the corresponding histological examination results. Histological examination of the specimens and evaluation of the corresponding dual fluorescence imaging were performed by a single expert gastrointestinal pathologist.

Mismatches between dual fluorescence images and H&E histological images were observed, because the sliced tissue sections used for histological examination could not be the surface where dual fluorescence imaging was performed. To overcome this mismatch, additional confocal fluorescence microcopy (CFM) imaging was performed on the surface of tissue specimens after fluorescence labeling of the cell nucleus and cytoplasm, and CFM images were used as virtual histology for the comparison with dual fluorescence images. DAPI (4'6-diamidino-2-phenylindole) and eosin were used for the labeling of nucleus and cytoplasm, respectively. In the CFM, two lasers of 405 nm and 488 nm in wavelength were used to excite DAPI and eosin, respectively. Emission light of DAPI and eosin was spectrally resolved by splitting from 420 nm to 470 nm and from 570 nm to 650 nm, respectively. A 25x objective lens (HCX APO L, Leica) was used for CFM imaging [21]. CFM imaging had a FOV of 550 μm x 550 μm, and CFM images acquired at different lateral positions were combined. The CFM mosaic images were compared with dual fluorescence images.

Quantitative similarity analysis of dual fluorescence imaging

For the clinical application of dual fluorescence imaging, quantitative analysis was performed, and the similarity between moxifloxacin and proflavine imaging was calculated. Dual fluorescence imaging with 1.1 x 1.1 mm FOV were divided into 2 x 2 patches (0.55 mm x 0.55 mm FOV), and the correlation coefficient between the moxifloxacin and proflavine images in each image patch was calculated (Figure 2). A threshold value was calculated to maximize the classification of dual fluorescence imaging with reference to histological examination, and then accuracy analysis was performed.

Figure 2. Quantitative similarity analysis for dual fluorescence imaging



Dual fluorescence images were cropped to small size patches of 550 μm x 550 μm in FOV. The correlation between moxifloxacin (G) and proflavine (R) image patches was calculated. Correlation values from multiple image patches were plotted in histogram.

Results

Patients

During the study period, ten colonic and six gastric lesions from eight and four patients, respectively, were evaluated. The mean age of the enrolled patients was 66.9 years, and seven (58.3%) were women; half of the patients had at least one comorbidity. There were no immediate complications during the endoscopic procedures. Based on the final histopathological results of the resected lesions, 1 normal tissue, 8 adenomas, and 1 carcinoma were found in the colonic lesions, while 1 normal tissue, 2 adenomas, and 3 carcinomas were found in the gastric lesions, respectively (Table 1).

Table 1. Summary of the enrolled patients and relevant lesions

Case	Age	Sex	Comorbidity	Location of the lesion	Biopsy (before procedure)	Histopathology of the imaged specimen	Procedure Method	Final histopathology of the entire resected lesion (after procedure)
C0*	73	F	None	Colon, descending	None	Normal colonic mucosa	Forceps	Normal colonic mucosa
C1	74	F	None	Colon, transverse	None	Serrated adenoma	ESD	Traditional serrated adenoma
C2*	67	F	DM, HT	Colon, sigmoid	Villous adenoma, low-grade dysplasia	Tubular adenoma	EMR	Intramucosal adenocarcinoma in tubular adenoma
C3*#	66	F	None	Colon, cecum	None	Tubular adenoma	EMR	Tubular adenoma, low-grade dysplasia
C4	72	M	HT, gout, BPH	Colon, ascending	Tubular adenoma, low-grade dysplasia	NA	ESD	Tubular adenoma, low-grade dysplasia
C5	55	M	None	Colon, ascending	None	NA	EMR	Tubular adenoma, low-grade dysplasia
C6	62	F	HT	Colon,	Tubular adenoma, low-	NA	EMR	Tubular adenoma, low-grade dysplasia

				ascending	grade dysplasia			
C7*#	62	M	CAD, HT, DL	Colon, ascending	Tubular adenoma, low-grade dysplasia	NA	EMR	Tubular adenoma, low grade dysplasia
S0*	80	F	None	Stomach, antrum	None	Normal gastric mucosa	Forceps	Normal gastric mucosa
S1*#	64	M	DM, HT	Stomach, high body greater curvature	Tubular adenoma, low-grade dysplasia	Tubular adenoma	ESD	Tubular adenoma, low-grade dysplasia
S2	51	F	None	Stomach, angle lesser curvature	Tubular adenocarcinoma, well differentiated	Tubular adenoma	ESD	Tubular adenocarcinoma, well differentiated, pT1a
S3*#	77	M	Prostate cancer, HT	Stomach, antrum greater curvature	Tubular adenoma, focal high-grade dysplasia	Tubular adenoma	ESD	Tubular adenocarcinoma, moderately differentiated, pT1a

** These lesions were included in the qualitative similarity analysis.*

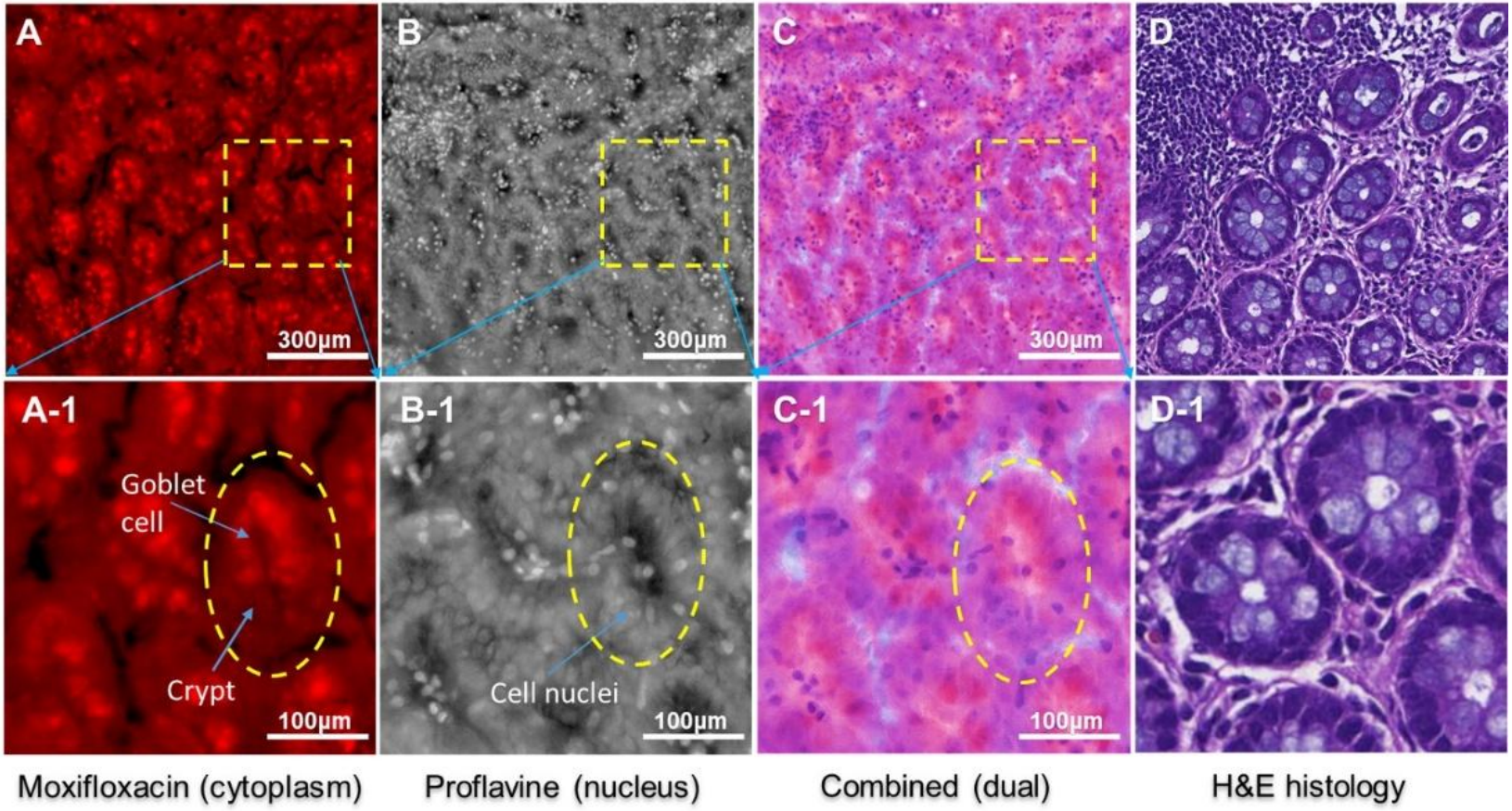
 # Two lesions in these patients were included in the qualitative analysis.

BPH, benign prostate hyperplasia; CAD, coronary artery disease; DM, diabetes mellitus; EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection; NA, not available; HT, hypertension

Normal colonic tissue

In a normal colonic tissue, moxifloxacin imaging showed normal colonic epithelial cells with preserved regular crypt structures on the surface. Goblet cells in the crypts were visualized with strong moxifloxacin expression compared to epithelial cells. Proflavine imaging showed a regular-shaped nuclei distributed along the outer boundary of the crypts. Dual fluorescence imaging showed the organized distribution of epithelial cells with polarized nuclei and the presence of goblet cells in the crypts, which was consistent with histological examination (Figure 3).

Figure 3. Normal colonic mucosa (case C0)



Colonic lesions

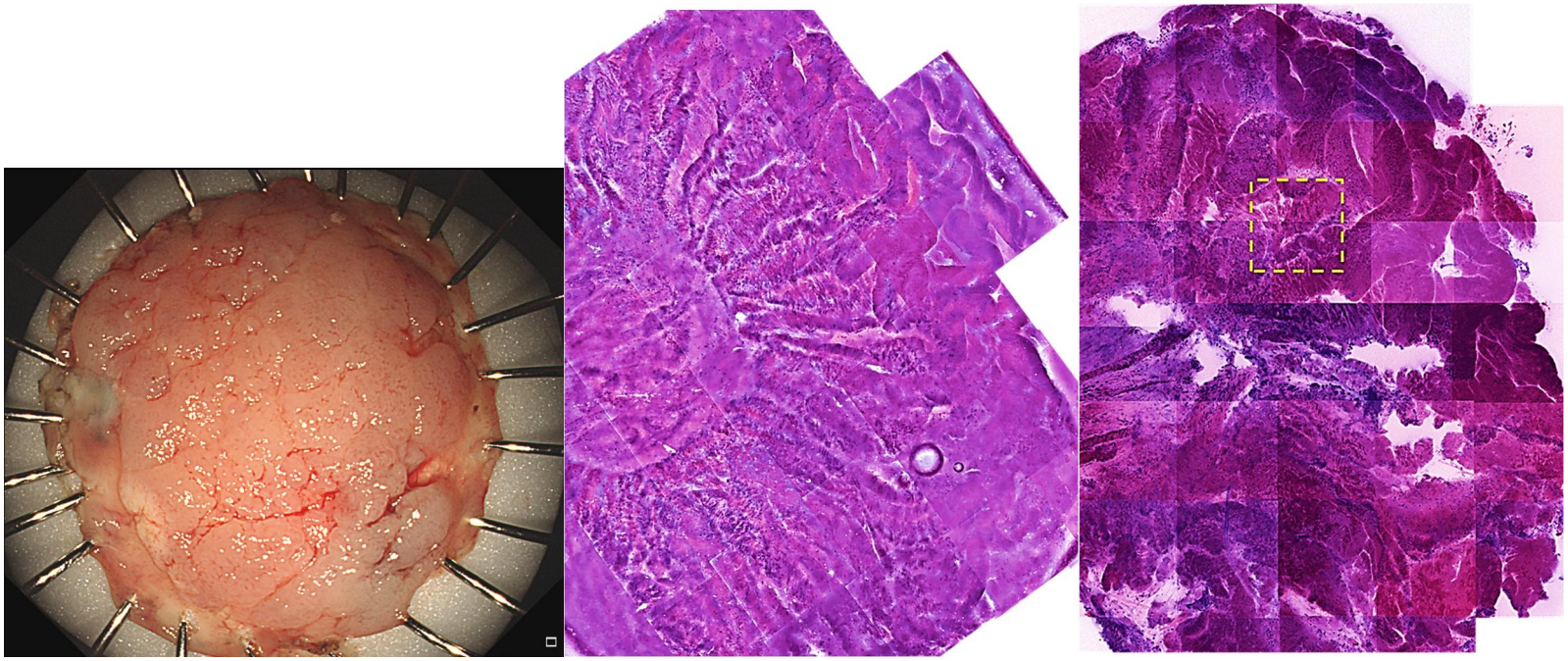
A total of nine colonic adenoma specimens were imaged. Representative dual fluorescence images of an adenoma specimen are shown in Figure 4. Separated images in moxifloxacin and proflavine channels, combined images in pseudo H&E colormap, and virtual and conventional H&E histological images are shown in two different FOVs. Moxifloxacin channel images showed irregular glands with disorganized or elongated cells, whereas elongated cell nuclei in the glands were observed in proflavine channel images. The combined images clearly showed an irregular nuclear distribution. The cellular and nuclear structures in the dual fluorescence imaging were consistent with the corresponding histology.

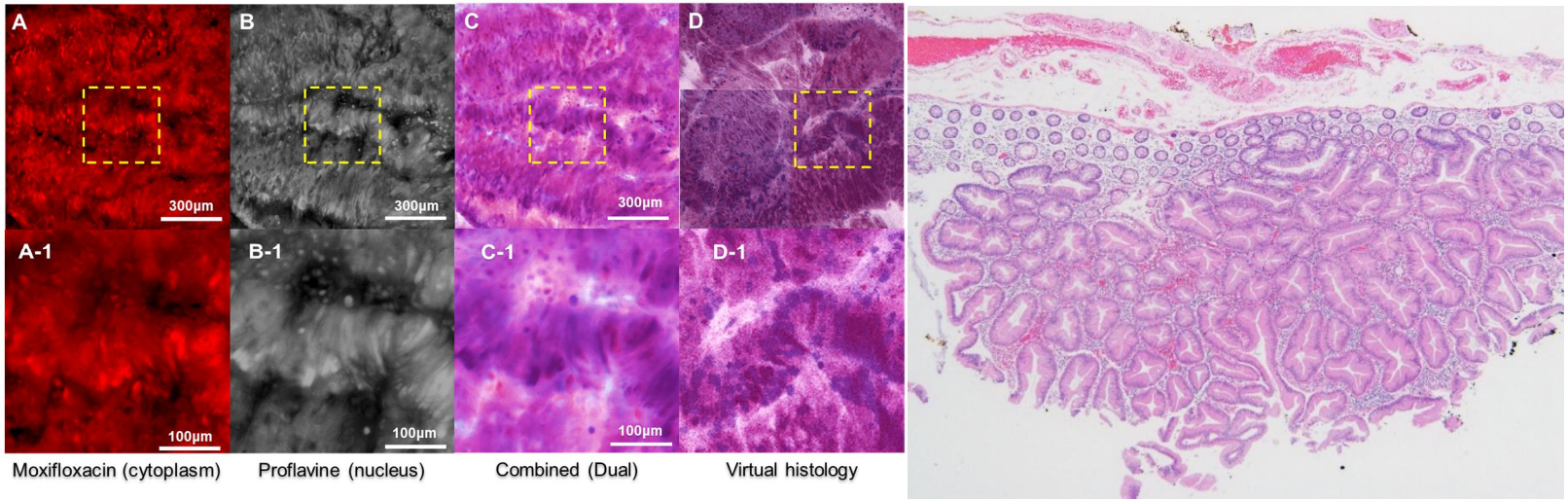
The dual images in separated moxifloxacin and proflavine channels, combined images in pseudo H&E colormap, and virtual histology images are shown in Figure 5. Moxifloxacin and proflavine channel images showed significantly distorted glands with elongated cells, and elongated cell nuclei in the distorted glands, respectively. The two channel images showed distorted glandular structures, while the cells in the glands were slightly different. To obtain an exact comparison between dual fluorescence imaging and histology, virtual histology was generated on the same surface. Virtual histology images, which are 3D confocal images on the specimen surface, showed distorted glands and elongated cell nuclei in the glands. The dual fluorescence images and virtual histology were consistent with each other. The specimen was fixed and fluorescently labeled with cell nucleus and cytoplasm dyes, and then imaged on the same surface with CFM. The acquired confocal images were displayed in pseudo H&E colormap.

The dual fluorescence imaging was conducted on the surface of specimens in fresh *ex vivo*

condition, while histological examination was processed after fixation and thin section. Therefore, dual fluorescence imaging could not be exactly matched with histology. The histologic images in Figure 6 were selected under the assumption that the gland structures in the specimen were similar to each other. There are some cells which are brightly depicted in the moxifloxacin channel compared to the proflavine channel. Goblet cells also appeared in the histology of the same specimen.

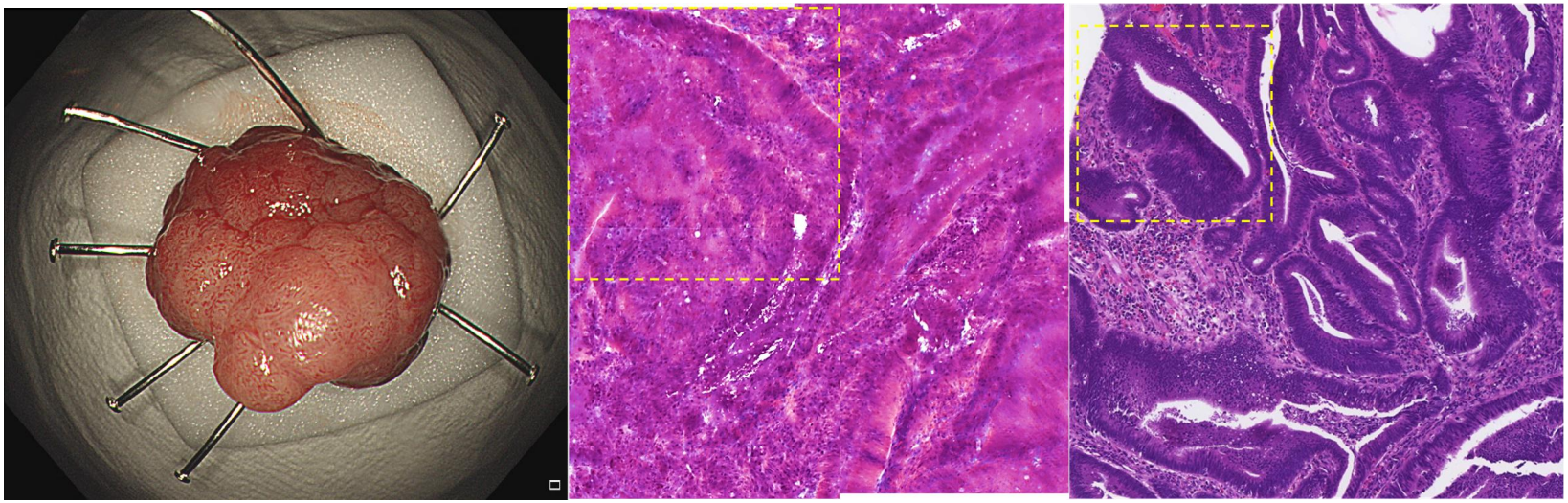
Figure 4. Colonic traditional serrated adenoma in the transverse colon (case C1).

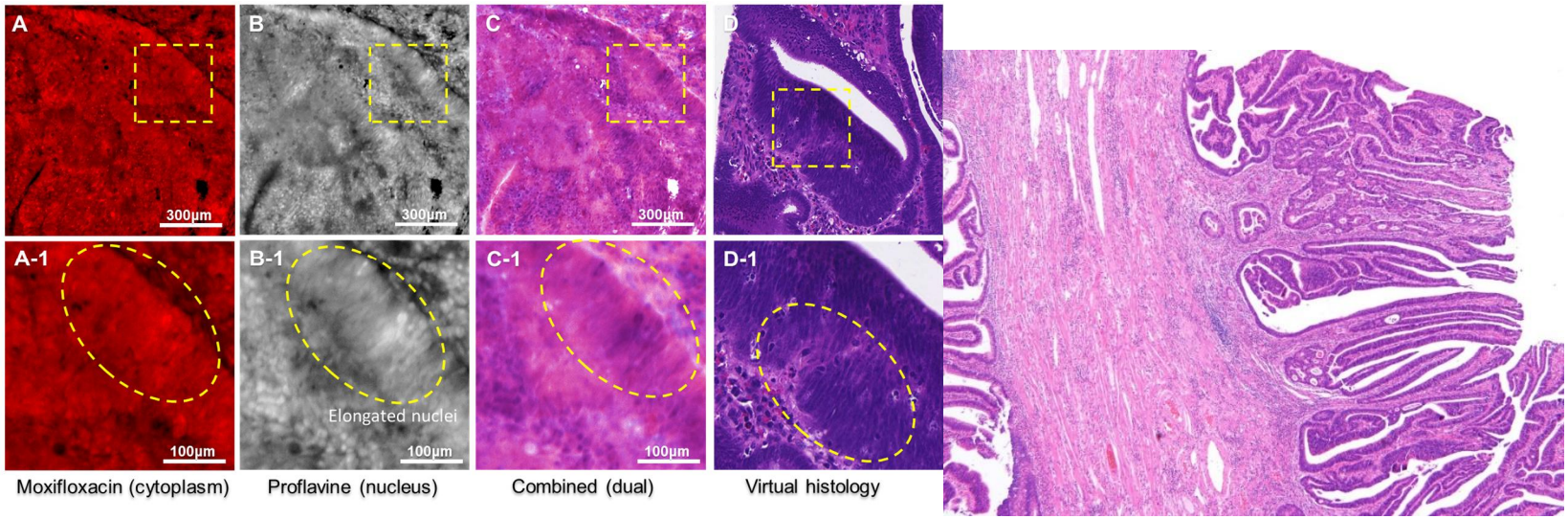




(upper left) grossly resected specimen, (upper middle) dual fluorescence imaging of sample, (right) confocal microscopic imaging of sample, (lower left) dual fluorescence imaging, (lower right) histological examination of grossly resected specimen

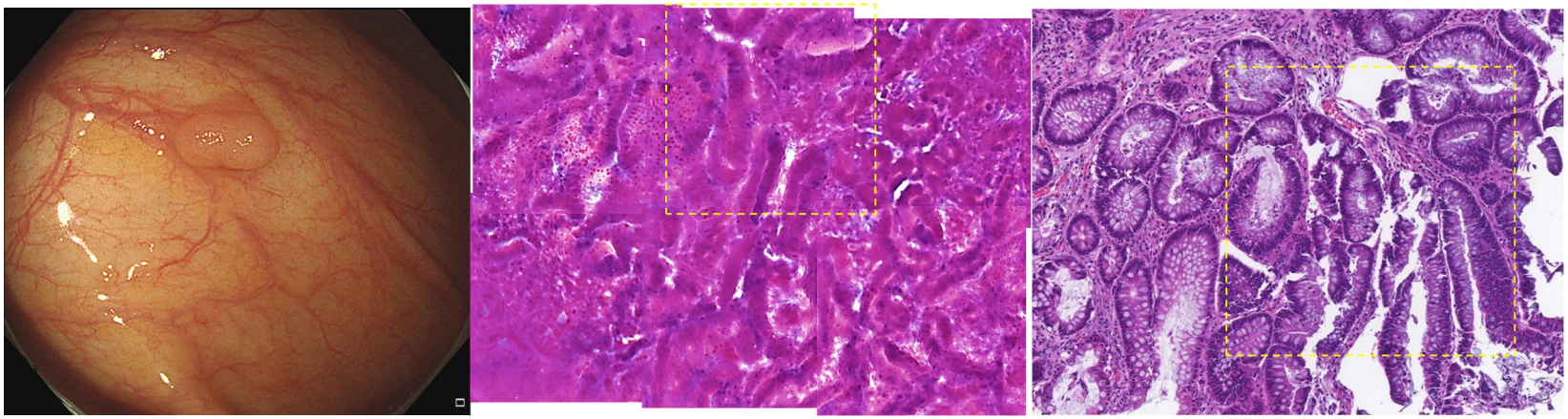
Figure 5. Colonic tubular adenoma with focal intramucosal adenocarcinoma in the sigmoid colon (case C2)

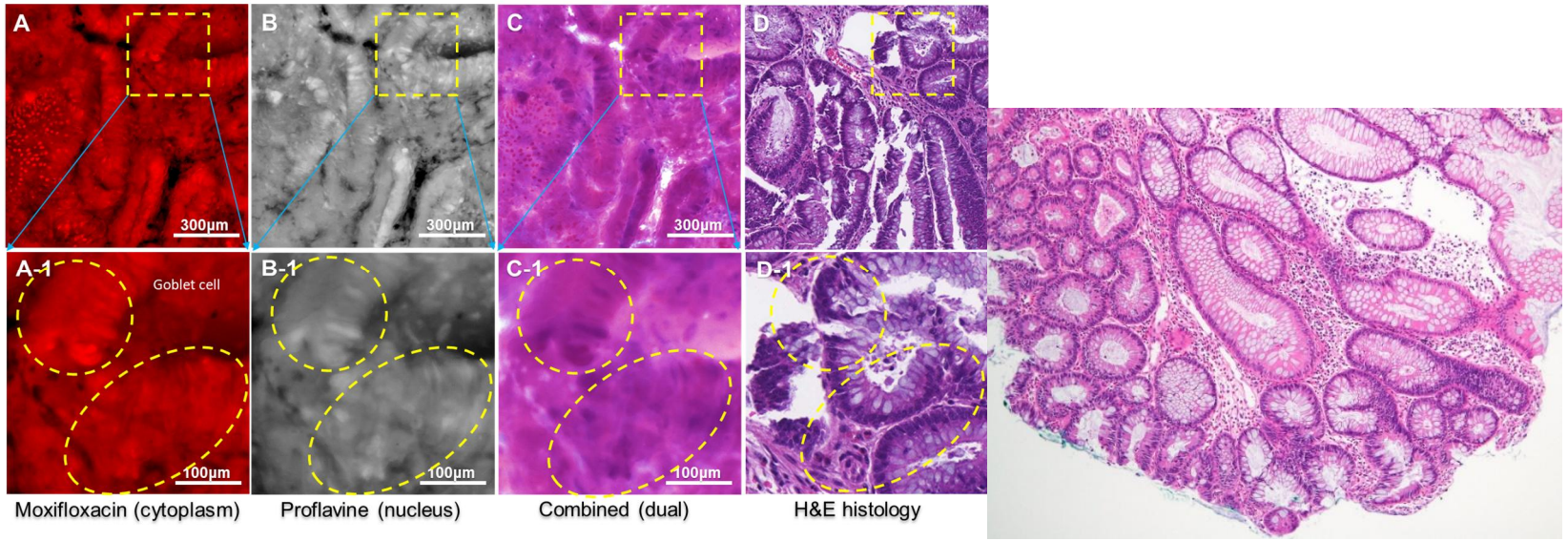




(upper left) grossly resected specimen, (upper middle) dual fluorescence imaging of sample, (right) histologic examination of sample, (lower left) dual fluorescence imaging, (lower right) histologic examination of grossly resected specimen

Figure 6. Colonic tubular adenoma with low-grade dysplasia in the cecum (case C3)



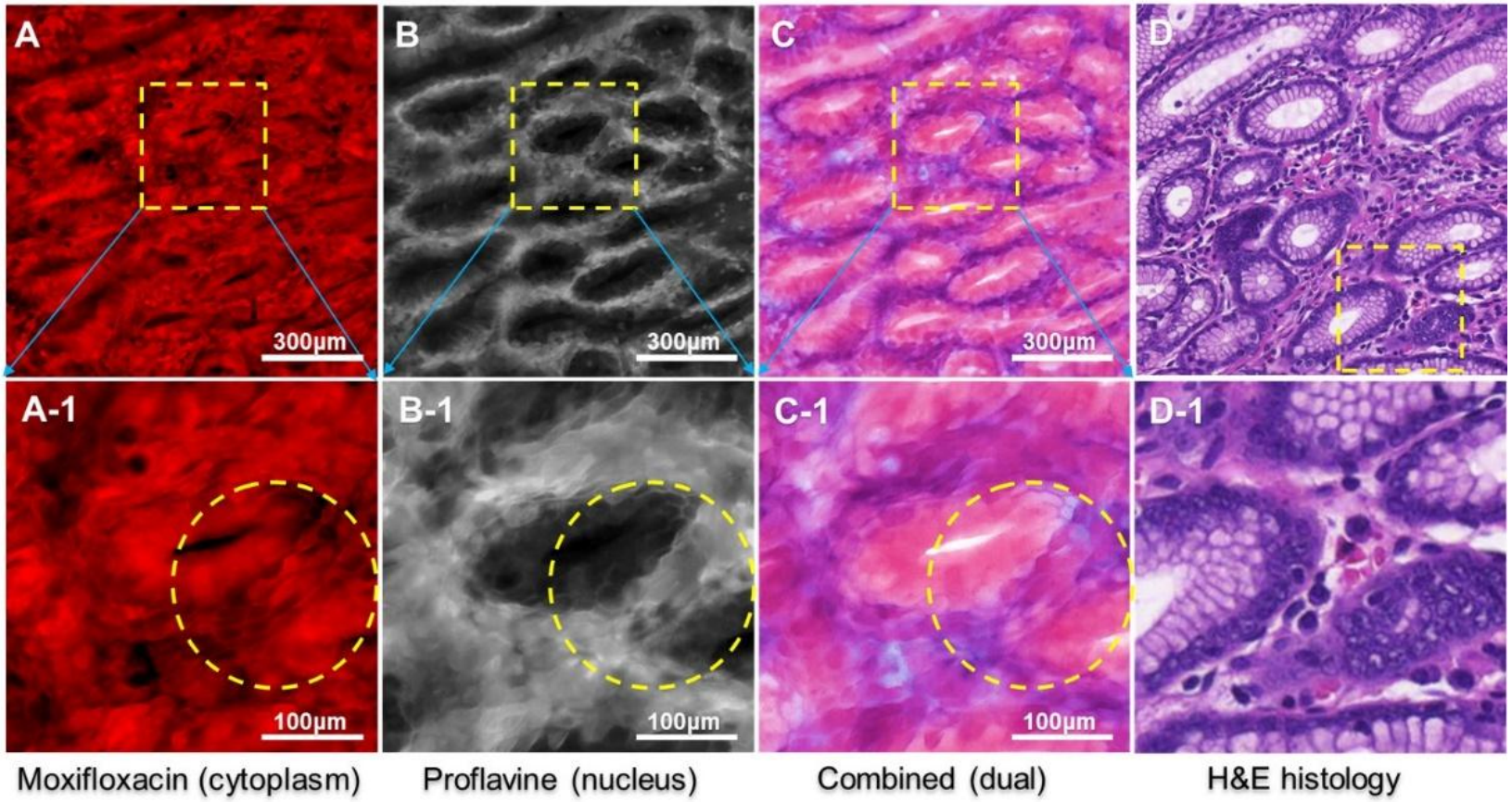


(upper left) gross lesion, (upper middle) confocal microscopic imaging of sample, (right) histologic examination of sample, (lower left) dual fluorescence imaging, (lower right) histologic examination of grossly resected specimen

Normal gastric tissues

In the normal gastric mucosa, moxifloxacin fluorescence imaging clearly showed cytoplasm inside the crypt, whereas proflavine fluorescence imaging showed nuclei outside the crypt. Dual fluorescence imaging clearly demonstrated the distribution of the regular pyloric gland and polarized nuclear arrangement, which was comparable with the histological examination (Figure 7).

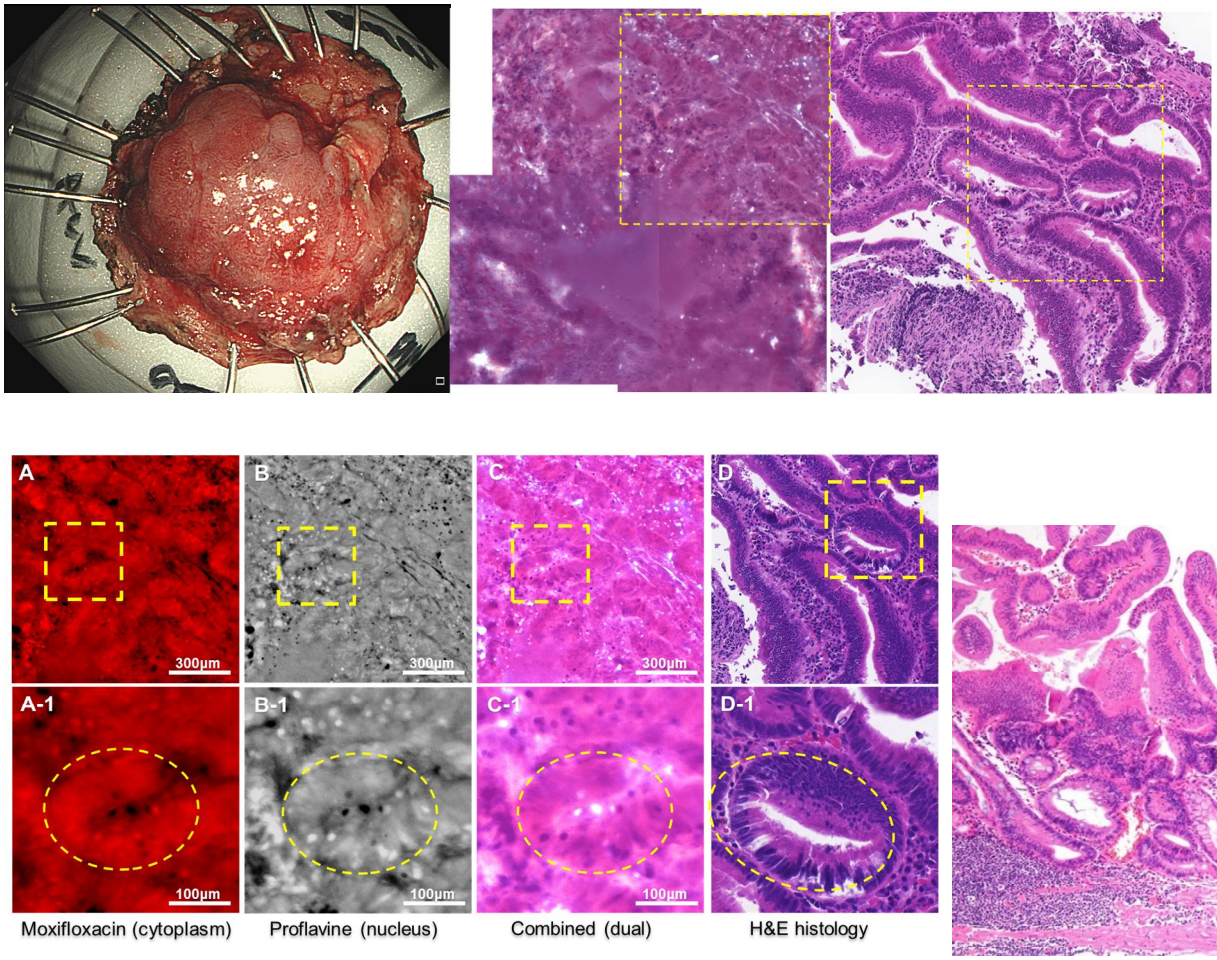
Figure 7. Normal gastric mucosa (case S0)



Gastric lesions

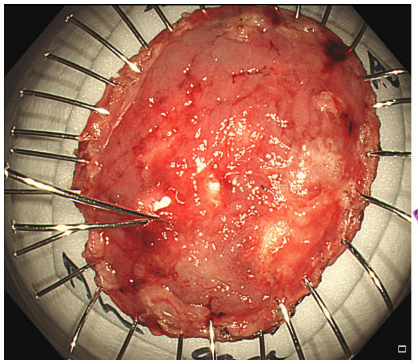
A total of five gastric adenoma or carcinoma specimens were imaged. Representative dual fluorescence images of the gastric lesions are shown in Figures 8-10. Separated images in moxifloxacin and proflavine channels, combined images in pseudo H&E colormap, and H&E histology images are shown in two different FOVs. Moxifloxacin channel images showed irregular glands with disorganized and elongated cells, while proflavine channel images showed densely scattered or elongated cell nuclei in the glands. Combined dual fluorescence imaging clearly showed nuclear distribution in the gland. The cellular and nuclear structures in dual fluorescence imaging were consistent with the corresponding histology. In the gastric adenoma or carcinoma, moxifloxacin fluorescence imaging showed irregular and unclear glands compared to the normal mucosa, while proflavine fluorescence imaging showed elongated and dispersed nuclei. Dual fluorescence imaging showed a compact gland with dysplastic nuclei and sparse cytoplasmic mucin. CFM imaging was comparable with histologic examination (Figure 9).

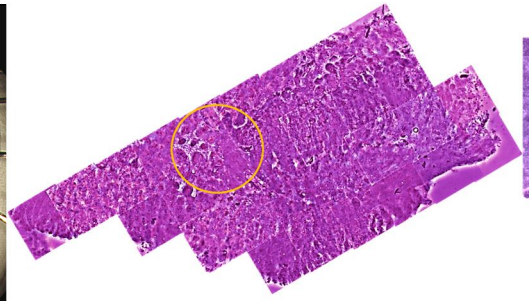
Figure 8. Gastric tubular adenoma in the body (case S1)



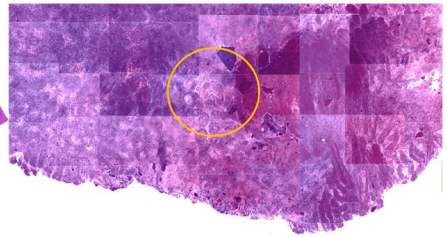
(upper left) grossly resected specimen, (upper middle) confocal microscopic imaging of sample, (upper right) histologic examination of sample, (lower left) dual fluorescence imaging, (lower right) histologic examination of grossly resected specimen

Figure 9. Well-differentiated gastric tubular adenocarcinoma in the antrum (case S2)

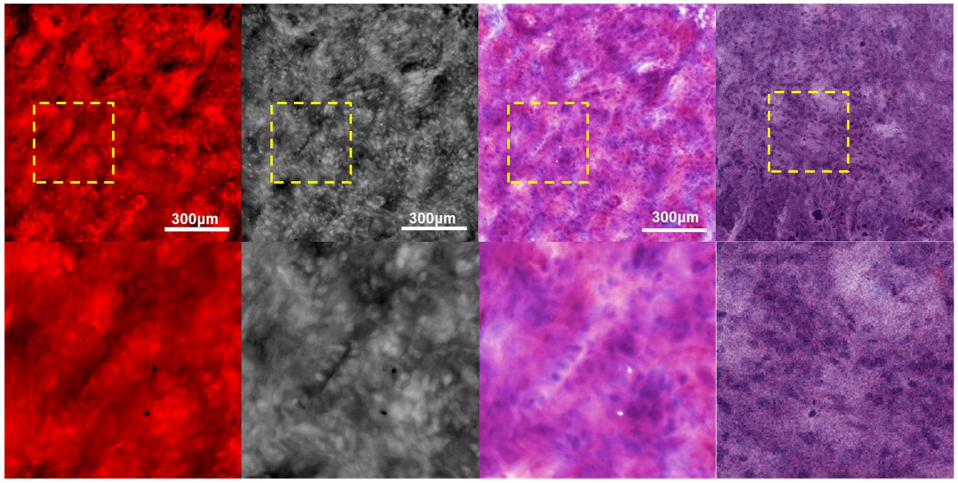




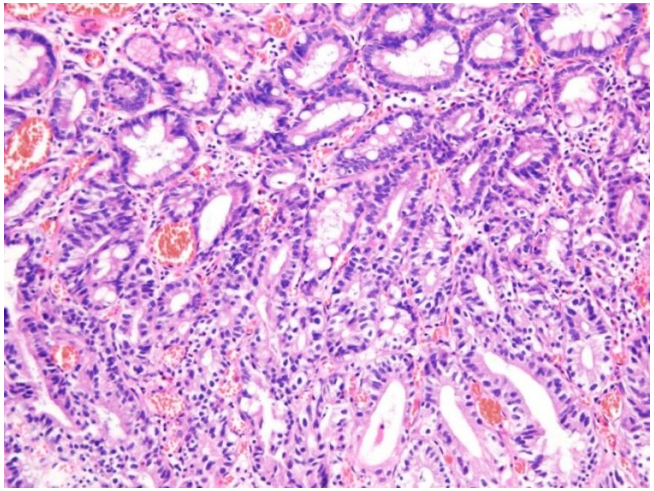
Moxifloxacin-Proflavine Dual fluorescence microscopy



Virtual histology, DAPI-Eosin confocal fluorescence microscopy

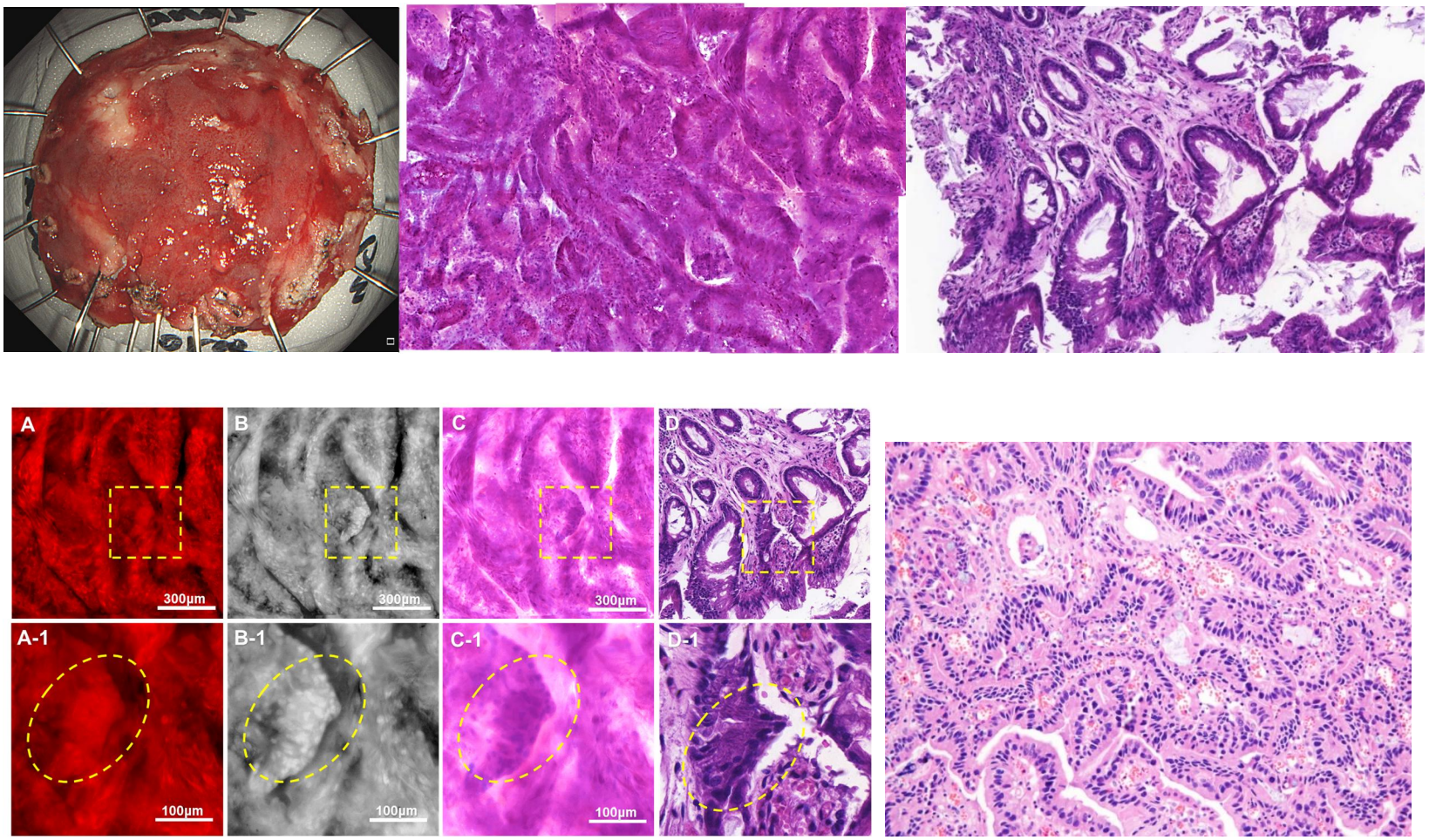


Moxifloxacin (cytoplasm) Proflavine (nucleus) Combined (dual) Virtual histology



(upper left) grossly resected specimen, (upper middle) gross dual fluorescence imaging of sample, (right) confocal microscopic imaging of sample, (lower left) dual fluorescence imaging, (lower right) histologic examination of grossly resected specimen

Figure 10. Moderately-differentiated gastric tubular adenocarcinoma in the antrum (case S3)



(upper left) grossly resected specimen, (upper middle) confocal microscopic imaging of sample, (upper right) histologic examination of sample, (lower left) dual fluorescence imaging, (lower right) histologic examination of grossly resected specimen

Quantitative similarity analysis of dual fluorescence imaging

A total of six and five colonic and gastric lesions in four and three patients, respectively, were included in the similarity analysis (Table 1). Compared with normal mucosa tissues, adenoma or carcinoma lesions showed high correlation values between moxifloxacin and proflavine imaging (Figure 11). When compared with histological examination, dual fluorescence imaging showed good accuracy in the colonic (82.3%) and gastric (86.0%) lesions at the threshold of 0.57 and 0.69, respectively (Table 2).

Figure 11. Quantitative similarity analysis between moxifloxacin and proflavine imaging

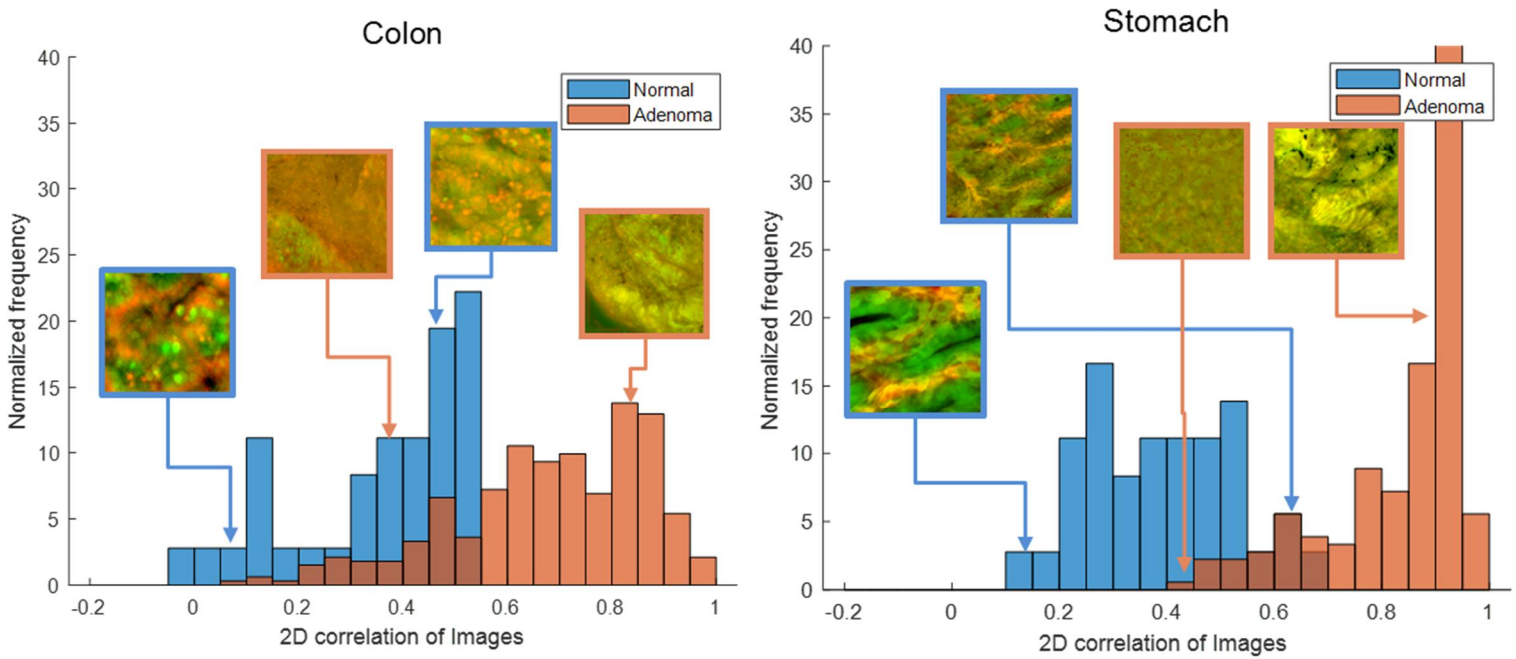


Table 2. Quantitative analysis of moxifloxacin and proflavine dual fluorescence imaging in the colonic and gastric lesions

	Colonic lesions	Gastric lesions
Accuracy	82.3%	86.0%
Threshold	0.57	0.69

Discussion

This is the first study to evaluate the feasibility of dual fluorescence imaging using moxifloxacin and proflavine in human gastrointestinal tract lesions. Dual fluorescence imaging was performed on the normal mucosa and premalignant or early cancerous lesion tissues of the colon and stomach. In the normal mucosa tissues, dual fluorescence imaging visualized normal glandular structures with polarized cell distribution. In the adenoma or carcinoma, abnormal glandular structures with elongated, disorganized cell nucleus comparable with histological examination were observed. In the similarity analysis comparing dual fluorescence imaging, adenoma or carcinoma lesion showed higher correlation values than that of the normal mucosa, suggesting its potential to aid in distinguishing neoplastic lesions from the normal mucosa.

Conventional diagnosis of gastrointestinal lesions is based on the visual observation of the lesion and following histological examination of the resected specimen. However, significantly high miss rates have been reported, which can vary depending on the experience of endoscopists [2,25-27]. In addition, the preparation process of histological examination samples inevitably causes time delay, and there is a possibility of false negative results due to sampling error during biopsy. Although the development of IEE technology enables real-time visual diagnosis in some parts, it is based on morphological changes of the surface and vessel as a result of histopathological progression to malignancy [28]. Thus, early neoplastic changes at the structural and cellular level can be missed, especially in the background of severe chronic inflammation (e.g., Barrett's esophagus, gastric intestinal metaplasia, and inflammatory bowel disease [IBD]) [29].

CLE shows high-magnification and high-resolution images of the mucosal layer in the

gastrointestinal tract, allowing real-time histologic diagnosis [11]. However, CLE has the inherent limitation of a small FOV, thus focusing on the focal lesion of interest and determining the overall lesion is difficult during endoscopic session [29]. In addition, the high cost of the instrument, long learning curve, and prolonged procedure time to obtaining clear images may prevent its widespread use. Regarding contrast for CLE, fluorescein cannot stain the nucleus; thus, additional nuclear staining is needed. In this regard, molecular imaging using specific targeting probes with wide-field endoscope showing molecular and functional aspects of the lesion is promising, although it has not been widely used until now [29-33]. In the era of personalized medicine, endoscopy providing real-time information about the exact tumor size, histopathological characteristics associated with poor prognosis such as lymphovascular or perineural invasion which increase the possibility of lymph node metastasis, are also promising. Thus, more specialized high-contrast high-resolution imaging methods would be helpful for the sensitive detection of gastrointestinal lesions.

In the present study, both moxifloxacin and proflavine were clinically applicable and topically instilled within a short staining time. Moxifloxacin is an Food and Drug Administration (FDA)-approved antibiotic, which has intrinsic fluorescence and good tissue penetration properties [22,23]. In a previous study, the mucosal tissue-to-serum ratio of moxifloxacin was 6.8 and 9.7 in the colon and the stomach, respectively, showing good mucosal penetration in the gastrointestinal mucosa of the patients [34]. The usefulness of moxifloxacin imaging has been demonstrated in other organs, especially in the conjunctiva, a goblet cell-rich organ [15,16,19]. Moreover, moxifloxacin has highlighted goblet cells five times more than AFI [17,21]. Based on these findings, we evaluated the clinical feasibility of moxifloxacin-based fluorescence endomicroscopy in the gastrointestinal tract

lesion prior to the present study. However, we found that moxifloxacin labeled cell cytoplasm and secretory cells well, whereas nuclear staining was lacking. Proflavine has fluorescent activity and specifically stains the nucleus. However, safety issues regarding many topical agents of fluorescence imaging (e.g., acriflavine) exist [11], and until now, proflavine is not approved by the FDA; thus, investigational drug approval is needed for use in human in previous studies. HRME using proflavine as a contrast agent could show detailed cellular imaging and structural changes [12,14]. Thus, in the present study, we could visualize both the cell nucleus and cytoplasm with dual fluorescence imaging using two different excitation wavelengths (moxifloxacin: 365 nm; proflavine: 455 nm) and similar emission wavelength around 500 nm by dedicated dual axially-swept wide-field microscopy, which obtained both moxifloxacin and proflavine fluorescence images in the same session (Figure 1).

Before the *in vivo* application of fluorescence imaging in daily practice, safety and efficacy of the contrast must be guaranteed. Moxifloxacin is advantageous in terms of safety as compared with other conventional imaging contrasts. Although proflavine is not an FDA-approved agent, there have been no reports of the significant adverse events, including mutagenic effects [1]. In the present study, we found that both moxifloxacin and proflavine have short incubation time for staining (< 5 min), but this should be verified *in vivo*. Another issue is the dedicated instrument and hardware that can be used during endoscopic procedures. In this study, we used dual axially-swept wide-field microscopy, but for *in vivo* dual fluorescence imaging, a dedicated endomicroscopic probe with sufficient working distance, which can be used through an endoscopic channel, is needed.

The maintenance of surface cell polarity is reported as a key feature of non-dysplastic epithelium in the gastrointestinal tract [35], and loss of polarity strongly suggests dysplastic

lesions in the colon [36] and stomach [37]. In dual fluorescence imaging, the distribution of cells and the presence or loss of cell polarity were well visualized and comparable with histological examination, suggesting that dual fluorescence imaging is feasible for detecting dysplastic structural and cellular changes in the colon and stomach before conventional histological examination. For example, dual fluorescence imaging can be used as an immediate *ex vivo* discriminator of complete resection in endoscopic or surgical resection specimen, especially in case of sessile serrated adenoma or colitis-associated neoplasm with longstanding IBD in the colon [38-40], or in case of signet ring cell or undifferentiated carcinoma in the stomach. Dual fluorescence imaging might be helpful in diagnosing neoplastic lesions in chronic inflammation affecting the goblet cell population. In a previous report, moxifloxacin-based fluorescence imaging clearly demonstrated goblet cell depletion in a dextran sodium sulfate-induced colitis mouse model [21]. In addition, dual fluorescence imaging can be useful in the prompt determination of adequate cellular quality after endoscopic ultrasound-guided fine needle aspiration biopsy, which can avoid unexpected additional procedure in another session or allow fewer number of needle pass.

In the similarity analysis, the correlation between moxifloxacin and proflavine images of adenoma or carcinoma was higher than that of the normal tissue (Figure 11). Moxifloxacin stains cytoplasm-rich cells (e.g., goblet cells), whereas proflavine specifically stains the cell nucleus. Moxifloxacin and proflavine images were varying in normal tissues owing to the polarized cell nucleus distribution and presence of goblet cells in the normal colonic tissue. In contrast, these two images were similar in the colonic and gastric lesions, although the reasons for such are unclear and thus need to be further investigated in the future.

This study has some limitations. First, there was a difference in the direction of view

between dual fluorescence imaging and histological examination, causing a mismatch between the horizontal fluorescence imaging and the vertical histological imaging. To overcome this issue, additional CFM was performed, which showed morphologic features similar to those of histologic examination. Second, in dual fluorescence imaging, neoplastic changes of the nucleus were observed mainly from proflavine imaging and not from moxifloxacin imaging. However, information about cytoplasmic staining, including goblet cells in the colon and cell distribution of the gland, can be derived from moxifloxacin imaging. For convenient clinical use of dual fluorescence imaging during endoscopy, the application of artificial intelligence can help to quickly process *in vivo* real-time diagnosis [41]. Third, because we only focused on precancerous or early cancer lesions of the colon and stomach, other common non-neoplastic lesions, such as hyperplastic or inflammatory polyp, esophageal lesions and chronic inflammation-associated lesions were not evaluated in this study. The number of enrolled cases was also relatively small. Thus, before the clinical use of dual fluorescence imaging, it should be verified in other lesions.

In conclusion, dual fluorescence imaging using moxifloxacin and proflavine was feasible in the evaluation of precancerous and early cancerous lesions in the colon and stomach. Glandular shape and epithelial cell organization, including goblet cells in the gland, were visualized in high contrast by dual fluorescence imaging. Both moxifloxacin and proflavine are safe contrast agents, which have fast fluorescence activity; thus, this high-contrast method is promising for *in vivo* real-time visual diagnosis. Further studies are required in order to develop a real-time diagnostic method.

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Glossary

AFI: autofluorescence imaging

BPH: benign prostate hyperplasia

CAD: coronary artery disease

CFM: confocal fluorescence microscopy

CLE: confocal laser endomicroscopy

DAPI: 4'6-diamidino-2-phenylindole

DM: diabetes

EMR: endoscopic mucosal resection

EC: endocytoscopy

ESD: endoscopic mucosal dissection

FDA: Food and Drug Administration

FOV: field of view

H&E: Hematoxyline & Eosin

HRME: high-resolution micro-endoscopy

HT: hypertension

IBD inflammatory bowel disease

IEE: image-enhanced endoscopy

LED: light emitting diodes

PBS: phosphate buffered saline

Abstract

Background and study aims: The development of endoscopic imaging enabled visual diagnosis during endoscopic sessions. High-contrast and high-resolution imaging technique may enable real-time sensitive detection of the lesions. This study aimed to investigate the feasibility of a novel dual fluorescence imaging using moxifloxacin and proflavine in premalignant and early cancerous lesions of the human gastrointestinal tract.

Patients and methods: Patients with premalignant and early cancerous lesions in the colon and stomach were prospectively enrolled in this study. The lesions were biopsied with forceps or endoscopically resected. Dual fluorescence imaging was performed with topical moxifloxacin and proflavine solutions and custom axially-swept wide-field fluorescence microscopy. Results were compared with conventional histological examination.

Results: Eight patients with ten colonic lesions (1 normal mucosa, 8 adenomas, 1 carcinoma) and four patients with six gastric lesions (1 normal mucosa, 3 adenomas, 3 carcinomas) were evaluated. Dual fluorescence imaging visualized detailed cellular morphology, arrangement, and structures. Regular glandular structures with polarized cell arrangement were observed in the normal mucosa tissue. Goblet cells were preserved in the normal colonic mucosa tissues. Irregular glandular structures with scanty cytoplasm and dispersed elongated nuclei were observed in the adenoma or carcinoma. Goblet cells were scarce or lost in the colonic lesions. Similarity analysis between moxifloxacin and proflavine imaging showed relatively higher correlation values in adenoma or carcinoma compared with those in normal mucosa. Dual fluorescence imaging showed good accuracy with histologic examination both in the colonic (82.3%) and gastric (86.0%) lesions.

Conclusions: High-contrast high-resolution dual fluorescence imaging using moxifloxacin and proflavine was feasible for obtaining detailed histological information in the gastrointestinal

premalignant and early cancerous lesions. Further studies are needed to develop dual fluorescence imaging as an *in vivo* real-time visual diagnostic method.

Key word: Microscopy; Gastrointestinal tract; Moxifloxacin; Proflavine; Precancerous condition