In Vivo Detection of Rat Colorectal Cancers by using a Dual-Wavelength Excitation Method

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Hypoxia is a characteristic feature of solid neoplasms, and insufficient oxygen supply increases cellular nicotinamide adenine dinucleotide (NADH) fluorescence, which is a main component of autofluorescence of the colorectal mucosa. We investigated whether a dual-wavelength excitation method which is optimized for sensing mucosal NADH fluorescence could be applicable to the detection of rat colorectal cancers in vivo. Rat colorectal adenocarcinomas were studied by using fluorescence stereomicroscopy. After autofluorescence images at 470 nm irradiated with dual-wavelength excitation at 365 nm (F365ex) and 405 nm (F405ex) were acquired, ratio images were produced by dividing F365ex by F405ex:

The excitation-emission wavelength pairs in F365ex and F405ex were adjusted for acquisition of NADH fluorescence and reference fluorescence. Based on observations from the luminal surface in vivo, F365ex/F405ex ratio images indicated a 1.57-fold higher signal value in the cancers than in the surrounding normal mucosa. The signal values in F365ex/F405ex ratio images were less mutually related with the hemoglobin concentration index. Small adenocarcinomas (less than 4 mm) could be detected on F365ex/F405ex ratio images. The results showed that NADH fluorescence measurement with little interference from tissue hemoglobin is efficient for visualizing rat colorectal cancers in vivo, suggesting that the dual-wavelength excitation method has potential for label-free endoscopic detection of diminutive colorectal neoplasms.

Key words: colorectal cancer, detection, fluorescence, in vivo, nicotinamide adenine dinucleotide

I. Introduction

Hypoxia is characteristic of primary solid neoplasms [12, 22]. Under hypoxic conditions, insufficient oxygen supply introduces changes of the redox ratios of metabolism-related cellular fluorophores, such as nicotinamide adenine dinucleotide (NADH). It is reported that fluorescence of NADH is increased in hypoxic cells [13, 14]. Epithelial cells in the colorectal mucosa abundantly contain NADH [5, 13, 15, 16, 20, 21]. Change in mucosal autofluorescence in the process of neoplastic transformation may be useful in detecting diminutive epithelial neoplasms in the colorectum.

We have previously reported that adenomatous

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mucosa could be distinguished from normal mucosa in endoscopically-resected ex-vivo human colorectal specimens by detecting alteration of NADH fluorescence in the mucosa [6]. In that study, we developed a dual-wavelength excitation method to valuate NADH fluorescence in colorectal adenomatous mucosa without apparent reliance on tissue hemoglobin. After autofluorescence images at 470 nm irradiated with dual-wavelength excitation at 365 nm (F_{365ex}/F_{405em}) and 405 nm (F_{405ex}) were serially obtained, F_{365ex}/F_{405em} ratio images were created: The excitation-emission wavelength pairs in F_{365ex} and F_{405em} were designed to acquire NADH fluorescence and reference fluorescence. F_{365ex}/F_{405em} ratio images made clearly visible diminutive colorectal adenomas without interference from gross morphology or changes of vascularization in endoscopic mucosal resection (EMR) specimens.

It has not been demonstrated, however, whether the dual-wavelength excitation method is also effective in detecting tumors in vivo before excision. The aim of our present study was to examine whether the dual-wavelength excitation method could be applicable for in-vivo detection of rat colorectal cancers. Since the method is postulated to detect alterations of metabolic changes in the mucosal layer, it is preferable to precisely study specimens under in-vivo conditions. Furthermore, our previous study used EMR specimens to verify the feasibility of the method. It seems to be important to examine bowel specimens consisting of all layers of the wall, since the previous study failed to absolutely exclude the possibility that this method is influenced by autofluorescence emitted from submucosal collagen. The results showed that the dual-wavelength excitation method was applicable to in-vivo detection of diminutive colorectal cancers in rats.

II. Materials and Methods

Experimental colorectal tumor model

A preclinical rat model of azoxymethane (AOM)-induced colorectal tumors was used for the experiments. The induction of colorectal tumors was carried out according to previously reported protocols [10, 18]. Briefly, six-week-old male F344 rats were subcutaneously injected with AOM (Sigma Chemical Co., St Louis, MO, USA) in sterile saline at a dose of 15 mg/kg of body weight once a week for 3 weeks. The rats were regularly fed after injection of AOM. After the sixth month following the beginning of AOM treatment, screening examinations of the descending colon and rectum by endoscopy (BF TYPE 3C40; Olympus, Tokyo, Japan) were performed for the assessment of the presence or absence of tumors. In the presence of tumors, the tumor-bearing rats were subjected to experiments. Tumors of the descending colon and rectum were used for the subsequent analysis. All experimental procedures were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine.

Sample preparation

In ex-vivo studies, the tumor-bearing rats were deeply anesthetized and their induced colorectal tumors with the surrounding non-tumorous tissues were quickly but carefully enucleated so as to prevent congestion and hypoxia. To minimize metabolic disintegration after resection, all samples were immediately immersed in physiologic saline at 4°C. In the cross-sectional study, colorectal tumors with surrounding non-tumor tissues were cut into slices with 1-mm-thickness and washed in physiologic saline. Cross-sectional specimens were prepared after acquisition of mucosal surface fluorescence images. The specimens were placed on a thermo plate kept at 3°C during experiments to keep them wet and fresh. All experiments were performed within 1 hour of excision.

In in-vivo experiments, the anesthetized rats were intubated with plastic tubes (outside diameter, 1.7 mm), and placed in the supine position. The intubated rats were artificially ventilated with room air. After abdominal midline incision, the anterior walls of the descending colon and rectum were cut open longitudinally with an electrosurgical knife. The exposed colorectal lumen was washed with saline at 37°C, and was scanned for colorectal tumors under white light imaging. The recognized colorectal tumor areas were then analyzed by a fluorescence stereomicroscopic system. The ventilated rats were placed on the thermo plate kept at 37°C during the experiment.

Microscopic imaging

A fluorescence stereomicroscopic imaging system previously described [6] was also used, with some modifications for this study. Briefly, the system for microscopic imaging consisted of a stereozoom microscope (SZX12; Olympus) equipped with a monochrome CCD camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan), a mercury lamp (U-LH100HG; Olympus), an objective lens (DF PLAPO 1.2× PF2; Olympus), and a thermo plate (MATS-555S; Tokai Hit, Fujinomiya, Japan). A halogen lamp (LG-PS2; Olympus) and a color CCD digital camera (DP71; Olympus) were also equipped with the microscope to examine the structure of the samples, and 365-nm and 405-nm band-pass filters (D365/10× and D405/20×; Chroma Technology, Rockingham, Vermont) were used for 365 nm and 405 nm excitations, respectively. A 470-nm band-pass filter (D470/40m; Chroma) was used as an emission filter. A 610-nm band-pass filter (610DF20; Omega Optical, Brattleboro, Vt) and a 550-nm band-pass filter (HQ550/20×; Chroma) were used to acquire reflected images. Autofluorescence images at 470 nm illuminated with dual-wavelength excitation light at 365 nm (F_{365ex}) and 405 nm (F_{405ex}), and reflection images at 550 nm (R_{550}) and 610 nm (R_{610}), were taken by using the monochrome CCD. Dark current images measured without incoming lights were subtracted from measured fluorescence and reflection images for analysis.
**Image analysis**

Image analysis was performed as described previously [6] with some modifications. Briefly, Image J software (National Institutes of Health, Bethesda, Maryland) was used for analysis. After acquisition of F$_{365\text{ex}}$ and F$_{405\text{ex}}$, ratio images were produced by dividing F$_{365\text{ex}}$ by F$_{405\text{ex}}$. Signal intensities of autofluorescence and reflected light were evaluated from the images obtained by the monochrome CCD. Regions of interests (ROIs) were set over neoplastic sites and neighboring normal mucosal sites as assessed based on pathological features of the samples. Average pixel or ratio values of ROIs were reckoned using Image J. Bias of the monochrome CCD and variant exposure time were compensated prior to evaluation of pixel values. Ratios between signal intensities in tumorous regions and normal regions in F$_{365\text{ex}}$ (T/N ratio in F$_{365\text{ex}}$), F$_{405\text{ex}}$ (T/N ratio in F$_{405\text{ex}}$), and F$_{365\text{ex}}$/F$_{405\text{ex}}$ ratio images (T/N ratio in F$_{365\text{ex}}$/F$_{405\text{ex}}$) were calculated. The sizes of tumors were defined by their maximum diameter measured from the color CCD images. Log(R$_{365}$/R$_{550}$) was adopted as a value for determining the amount of tissue hemoglobin, because the logarithm of the ratio of red reflectance to green reflectance is known as an estimating value for the tissue hemoglobin content [9, 19].

**Histopathology**

All of the tumor samples were subjected to pathologic examination after formalin fixation and paraffin embedding. Cross sections of 4 μm thickness were stained with hematoxylin-eosin (H&E). A pathologist conducted histopathological diagnosis without knowing the results of the fluorescence imaging.

**Statistical analysis**

The calculated signal intensity ratios between tumorous mucosa and normal mucosa were compared with 1.0 using a one-sample t-test. A probability value of less than 0.05 was considered significant. Data are given as means ± standard deviations.

**III. Results**

**Histopathology**

A total of 21 AOM-induced colorectal tumors from 21 rats were included in this study. All of the tumor lesions were recognized as protruding lesions, and diagnosed as tubular adenocarcinomas. The sizes of the lesions ranged from 2.0 to 9.0 mm (average, 5.15 mm).

**Dual-wavelength excitation imaging in colorectal cross-sections**

Firstly, autofluorescence from the mucosal layer was observed in cross-sections of rat colorectal specimens (n=10: 10 cross-sections from 10 tumor tissues with surrounding non-tumor tissues) under the stereomicroscope. Stereomicroscopic and histologic images in a representative cross-section of a colorectal adenocarcinoma are shown in Figure 1A–D. In F$_{365\text{ex}}$ and F$_{405\text{ex}}$, it is difficult to identify obviously tumorous mucosa on the basis of fluorescence intensity, because the differences of fluorescence intensity between tumorous mucosa and normal mucosa are small (Fig. 1B and C). However, the ratio images of F$_{365\text{ex}}$/F$_{405\text{ex}}$ showed tumorous lesions in accordance with the histologic picture, as shown in Figure 1A and 1D. To quantitatively analyze the fluorescence of tumorous and normal mucosa on cross-sectional observation, we examined the T/N ratios in F$_{365\text{ex}}$ F$_{405\text{ex}}$, and F$_{365\text{ex}}$/F$_{405\text{ex}}$ images (Fig. 1E).

The average T/N ratio in F$_{365\text{ex}}$ was more than 1.0 (1.40±0.073, p<0.01), and that in F$_{405\text{ex}}$ was less than 1.0 (0.81±0.13, p<0.01). There were still a few cases observed in which lesions were difficult to differentiate from normal areas owing to the small difference between the T/N ratios and 1.0 in the case of F$_{405\text{ex}}$. The average T/N ratio in F$_{365\text{ex}}$/F$_{405\text{ex}}$ demonstrated apparent increase (1.55±0.12, p<0.001), and all of the analyzed adenocarcinomas could be detected clearly using T/N ratio in F$_{365\text{ex}}$/F$_{405\text{ex}}$.

**Dual-wavelength excitation imaging from luminal surface in ex-vivo and in-vivo colorectums**

We examined whether the dual-wavelength excitation method is suitable for tumor detection from the luminal surface. Representative images of an ex-vivo specimen of adenocarcinoma observed from the luminal surface are shown in Figure 2A–E. In the observation from this surface, the tumorous lesion was clearly identified on the ratio images of F$_{365\text{ex}}$/F$_{405\text{ex}}$ (Fig. 2E). Figure 2F shows the quantitative results for T/N ratios in F$_{365\text{ex}}$, F$_{405\text{ex}}$, and F$_{365\text{ex}}$/F$_{405\text{ex}}$ when ex-vivo specimens were observed from the luminal surface (n=10: 10 tumors with surrounding non-tumor tissues from 10 rats). The average T/N ratios in both F$_{365\text{ex}}$ and F$_{405\text{ex}}$ were less than 1.0 (F$_{365\text{ex}}$: 0.90±0.22; F$_{405\text{ex}}$: 0.60±0.22). On the other hand, the average T/N ratio in F$_{365\text{ex}}$/F$_{405\text{ex}}$ demonstrated significant increase (1.62±0.20, p<0.001), and all adenocarcinomas analyzed could be detected using T/N ratio in F$_{365\text{ex}}$/F$_{405\text{ex}}$.

Then, we examined whether the method is also effective for tumor detection from the luminal surface in in-vivo samples (n=11: 11 tumors in 11 rats). Representative images of an in-vivo sample of adenocarcinoma observed from the luminal surface are shown in Figure 3A–C. The tumor lesion was clearly identified in the F$_{365\text{ex}}$/F$_{405\text{ex}}$ image (Fig. 3C). The quantitative results of the T/N ratios in F$_{365\text{ex}}$, F$_{405\text{ex}}$, and F$_{365\text{ex}}$/F$_{405\text{ex}}$ images are shown in Figure 3D. The average T/N ratios in both F$_{365\text{ex}}$ and F$_{405\text{ex}}$ were less than 1.0 (F$_{365\text{ex}}$: 0.89±0.48; F$_{405\text{ex}}$: 0.62±0.34). In contrast, the average T/N ratio in F$_{365\text{ex}}$/F$_{405\text{ex}}$ was 1.57±0.28 (p<0.01) (Fig. 3D), and all of the samples analyzed were also detectable using T/N ratio in F$_{365\text{ex}}$/F$_{405\text{ex}}$.

**Influence of hemoglobin concentration on autofluorescence intensity in-vivo**

To analyze the influence of blood volume on tissue
autofluorescence in vivo, signal values of non-tumoral mucosal regions in F\textsubscript{365ex}, F\textsubscript{405ex}, and F\textsubscript{365ex}/F\textsubscript{405ex} ratio images were compared with log(R\textsubscript{610}/R\textsubscript{550}), an index of hemoglobin concentration: R\textsubscript{550} and R\textsubscript{610} represent reflection intensities at 550 nm and 610 nm, respectively (n=12: 12 regions in 6 rats). The signal intensities in F\textsubscript{365ex} and F\textsubscript{405ex} tended to decrease as log(R\textsubscript{610}/R\textsubscript{550}) increased (Fig. 4A and B). The slopes of the linear fitting curves in F\textsubscript{365ex} and F\textsubscript{405ex} were −2.1 and −3.2, respectively. However, the signal values in F\textsubscript{365ex}/F\textsubscript{405ex} ratio images were less mutually related with log(R\textsubscript{610}/R\textsubscript{550}) (Fig. 4C). The slope of the linear fitting curve in F\textsubscript{365ex}/F\textsubscript{405ex} (−0.7) was smaller than those in F\textsubscript{365ex} and F\textsubscript{405ex}. The results showed that the signal intensities in F\textsubscript{365ex}/F\textsubscript{405ex} ratio images were less affected by blood volume contained in in-vivo tissues.

**Relation between T/N ratio in F\textsubscript{365ex}/F\textsubscript{405ex} and sizes of adenocarcinomas**

The T/N ratio in F\textsubscript{365ex}/F\textsubscript{405ex} on luminal observation was compared with the sizes of the neoplasms to evaluate its validity in visualizing small neoplasms. Figure 5 shows the relation between T/N ratio in F\textsubscript{365ex}/F\textsubscript{405ex} and maximum diameter of both ex-vivo (black dots) and in-vivo (white dots) adenocarcinomas. The results showed that small adenocarcinomas less than 4 mm in diameter were well-discriminated in F\textsubscript{365ex}/F\textsubscript{405ex} ratio images even in in-vivo experiments.

**IV. Discussion**

In this study, we examined the efficacy of the dual-
wavelength excitation method to detect rat colorectal adenocarcinomas in vivo. To address this issue, we firstly elucidated the mucosal fluorescence intensities in cross-sections of colorectal cancers. Cross-sectional samples were used to analyze the neoplastic/non-neoplastic mucosal fluorescence without the overlay of submucosal fluorescence or the influence of mucosal thickness. By using the dual-wavelength excitation method, we found that all of the cancerous mucosae examined could be distinguished from non-cancerous mucosae in the cross-sectional samples.

It is important to examine whether the dual-wavelength excitation method can be applied for cancer detection from the luminal surface in consideration of the possible future application to endoscopic examination. The colorectal wall constitutes a multilayered structure, and in addition to the metabolic fluorophores mainly distributed in the mucosal layers, other bright endogenous fluorophores, such as collagen and elastin, are contained in the submucosal layers in the colorectum [7, 21]. The results showed that the dual-wavelength excitation method was also effective for cancer detection under observation from the luminal surface under not only ex-vivo but also in-vivo...
Dual-wavelength excitation method can identify cancerous lesion in the observation from the luminal surface in-vivo. Representative images of a colorectal adenocarcinoma are shown (A–C): A white-light image (A), an H&E-stained image (B), and a pseudo-color ratio image (C) obtained by dividing $F_{365ex}$ by $F_{405ex}$. Different colors in C represent different ratios as indicated by the inserted color bars. Bar=1 mm (A). Scatter plots for the T/N ratios of $F_{365ex}$, $F_{405ex}$, and $F_{365ex}/F_{405ex}$ in in-vivo samples are shown in D. The short horizontal lines show average values.

Fig. 3. Association between fluorescence intensity and hemoglobin concentration in-vivo. The relation of $F_{365ex}$ signal intensities to log($R_{610}/R_{550}$) (A), $F_{405ex}$ signal intensities to log($R_{610}/R_{550}$) (B), and $F_{365ex}/F_{405ex}$ signal intensities to log($R_{610}/R_{550}$) (C) were measured for in-vivo specimens on luminal observation. No significant correlation between $F_{365ex}/F_{405ex}$ and log($R_{610}/R_{550}$) [r (correlation coefficient) = −0.17 (C)] was observed. $R_{610}$ and $R_{550}$ represent reflection intensities at 610 nm and 550 nm, respectively. a.u., arbitrary units.

Fig. 4. Association between fluorescence intensity and hemoglobin concentration in-vivo. The relation of $F_{365ex}$ signal intensities to log($R_{610}/R_{550}$) (A), $F_{405ex}$ signal intensities to log($R_{610}/R_{550}$) (B), and $F_{365ex}/F_{405ex}$ signal intensities to log($R_{610}/R_{550}$) (C) were measured for in-vivo specimens on luminal observation. No significant correlation between $F_{365ex}/F_{405ex}$ and log($R_{610}/R_{550}$) [r (correlation coefficient) = −0.17 (C)] was observed. $R_{610}$ and $R_{550}$ represent reflection intensities at 610 nm and 550 nm, respectively. a.u., arbitrary units.
conditions. Fluorescence signals of the mucosa were not overwhelmed by those of the submucosa even under the in-vivo condition.

Our results demonstrated that the average T/N ratio in F \textsubscript{365ex}, which was acquired by a single-wavelength excitation light, was more than 1.0 in ex-vivo cross-sectional specimens in a reflection of the strong NADH fluorescence intensity in cancerous lesions (Fig. 1E). However, the average T/N ratio in F \textsubscript{365ex} in in-vivo specimens was less than 1.0, as shown in Figure 3D. This may be partly because the ex-vivo specimens seemed to have a lower blood content than the in-vivo specimens: In the ex-vivo specimens cut into slices with 1-mm-thickness, blood in the tissues was considered to have oozed out in physiologic saline during the specimen-making process, and blood hemoglobin absorbs both excitation and emission light of NADH [4, 6]. In the dual-wavelength excitation method, the reference fluorescence could compensate for the absorption effect of hemoglobin. Indeed, the signal intensities in F \textsubscript{365ex}/F \textsubscript{405ex} ratio images were less affected by blood hemoglobin in the in-vivo samples (Fig. 4C). Therefore, the method seemed to be suitable for in-vivo detection of cancerous lesions based on NADH fluorescence. As for the reference fluorescence, the main fluorophores responsible for the reference fluorescence may be flavoproteins [5, 6, 21]. Flavoprotein fluorescence is reported to show behavior inverse to NADH fluorescence in cells [11, 17, 21]. No effects on T/N ratio in F \textsubscript{365ex}/F \textsubscript{405ex} ratio images from the tumor site or degree of tumor differentiation were apparent in this study.

The ratio imaging using two excitation lights is useful for shading correction on three-dimensional objects such as hollow organs. Structures near the light source or in a plane perpendicular to the light axis tend to show strong signals. Conversely, structures far from the light source or in a plane parallel to the light axis tend to show dark signals. Such uneven illumination using a single excitation light in the gastrointestinal lumen can possibly lead to false detection of colorectal tumors. Our dual-wavelength excitation method can compensate for the effect of uneven illumination by dividing two images obtained with two excitation lights, as shown in Figures 1, 2, and 3. Therefore, the dual-wavelength excitation method may have potential for future development of a new device for automated identification of colorectal tumors.

Among the limitations of this study, firstly, we studied a rat model of colorectal cancers, not human colorectal cancers. It seems to be difficult to precisely study autofluorescence characteristics by using surgically-resected human colorectal cancer samples or human cancer specimens from endoscopic submucosal dissection, especially when focusing on metabolic fluorophores, because such samples, excised by relatively prolonged invasive treatments with hemostasis, are commonly in a severely ischemic state, i.e., hypoxic state. Secondly, we only found the histology of tubular adenocarcinoma in this experiment, and we studied neither colorectal cancer specimens coexistent with inflammatory bowel disease nor aberrant crypt foci. Future in-vivo research on human tumors with various histological types or with inflammatory bowel diseases is needed to verify the efficacy of this method in more depth. Thirdly, flat-type neoplastic lesions were not included in this study, although this method seems to have a potential to distinguish flat-type diminutive neoplastic lesions as discussed in the previous study [6]. In this study, we examined cross-sections of rat colorectal adenocarcinomas in order to demonstrate that this method was little-affected by the configuration of the specimens. Finally, the UVA light illumination has a potential to be harmful to the tissues. However, an optical implement to survey cervical neoplasms utilizing a UV light has been clinically applied [1, 2, 8]. Although it necessitates careful estimation of the use of UVA light, the brief use of UVA light at around 400 nm could be utilized on clinical inspection [3].

In our present study, we examined the efficacy of the

Fig. 5. Relation between T/N ratios in F \textsubscript{365ex}/F \textsubscript{405ex} when observed from the luminal surface and tumor size. T/N ratio in F \textsubscript{365ex}/F \textsubscript{405ex} and the sizes of tumors were compared. Adenocarcinomas less than 4 mm in diameter demonstrated high T/N ratios in F \textsubscript{365ex}/F \textsubscript{405ex}, as did large adenocarcinomas. Black and white dots indicate the results of ex-vivo (n=10) and in-vivo (n=11) samples.
The dual-wavelength excitation method to detect rat colorectal adenocarcinomas in vivo. Although in-vivo investigation using human colorectal neoplasms is indeed necessary, our results suggest the potential of the dual-wavelength excitation method as a label-free imaging technique of detecting diminutive neoplasms, which may be contributory to improving the usefulness of autofluorescence endoscopy of digestive tracts.

V. Abbreviations

AOM, azoxymethane; EMR, endoscopic mucosal resection; H&E, hematoxylin-eosin; NADH, nicotinamide adenine dinucleotide; ROI, regions of interest

VI. Acknowledgments

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