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ORIGINAL RESEARCH ARTICLE

All-in-one Raman spectroscopy approach to diagnosis of colorectal cancer: analysis of spectra in the fingerprint regions

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Abstract:

Objectives: Raman spectroscopy yields precise information, not only regarding the secondary structure of proteins but also regarding the discrimination between normal and malignant tissues. There is, however, no standard measurement method. We evaluated the use of a miniaturized, handheld, all-in-one Raman spectrometer with a 1064-nm laser excitation source for the diagnosis of colorectal cancer. The ultimate goal is real-time, in vivo diagnosis. Methods: Tissue samples were obtained from 20 patients who underwent surgery for colorectal cancer. The samples were irradiated with the portable Progeny™ Raman spectrometer, with which the Raman spectra were also obtained. We searched for characteristic Raman shifts and examined whether these shifts could distinguish the cancer tissues. To improve accuracy, we divided the spectra into 100 cm^{-1} bands and applied principal component analysis (PCA) to each range. We evaluated the contribution of each range for cancer discrimination. Results: Intensities at 1261 and 1427 cm^{-1} differed significantly between the normal tissues and cancer tissues, but these did not efficiently discriminate the cancer tissues. However, we were able to identify the characteristic spectral range in fingerprint regions; accuracy was 85.1%. Conclusions: Use of the all-in-one type Raman spectrometer can efficiently discriminate colorectal cancer, not on the basis of the intensities at 1261 and 1427 cm^{-1} but rather on the basis of PCA. Thus, Raman spectroscopy performed using a handheld device has potential to become a clinically powerful tool for producing high-quality data, obtaining highly reproducible measurements, and thus accurately diagnosing colorectal cancer.

Keywords:

all-in-one, colorectal cancer, Raman spectroscopy, 1064-nm excitation

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Introduction

The overall incidence of colorectal cancer has tended to rise; thus, in Japan, the number of patients with colorectal cancer is expected to increase[1)]. The associated mortality has also tended to rise, making early diagnosis and precise treatment clinically important for improving the prognosis and increasing the likelihood of cure. At present, histologic examination is the gold standard for diagnosis. However, this diagnostic method is invasive, time consuming, and costly, and it requires removal of tissue samples, which can be even unnecessary. New diagnostic methods that are minimally invasive, rapid, objective, and not prohibitively expensive are needed for the diagnosis of colorectal cancer in the early

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stage.

Raman spectroscopy is a powerful label-free modality that measures scattered light and can provide valuable structural information about materials and biological samples. When laser light is projected onto a sample, some scattered light is observed as Raman spectra. The scattered light is frequency-shifted with respect to the excitation frequency, and the frequency shift, referred to as the Raman shift, is directly related to the molecular structure of the sample. This principle was originally discovered in 1928 by Indian physicist C.V. Raman[2]. Raman spectroscopy was first applied in the fields of chemistry and mineralogy and in the study of semiconductors[3,4], However, it has recently been applied in the fields of biology and biochemistry because important information can be obtained regarding, for example, such macromolecules as nucleic acids, proteins, and lipids[5]. Raman spectroscopy has been used to identify the chemical species present in biological samples, to detect subtle changes in cells, and to discriminate various cancer tissues. Raman spectroscopy has shown potential for diagnosis of lung, esophagus, stomach, and breast cancers[6,7].

Tissues can be characterized in real time based on in vivo Raman spectra, and thus, Raman spectroscopy holds promise for real-time diagnosis of colon cancer. Shim et al.[8] reported clinical (in vivo) measurement of the Raman spectra of gastrointestinal tissues, and Molckovsky and colleagues compared Raman spectra from hyperplastic and adenomatous polyps in the colon and used principal component and discriminant analyses to develop diagnostic algorithms based on their spectral characteristics[9]. Bergholt et al.[10] reported the application of Raman spectroscopy for the diagnosis of adenomatous polyps during colonoscopy, and Stone et al.[11] reported the application of Raman spectroscopy in ex vivo studies of tissue samples of colorectal cancer. The diagnostic sensitivity in both studies was approximately 90%. Using an in-house-developed Raman probe for measurement and support vector machines as discriminative classifiers, Widjaja et al.[12] classified, at approximately 99% accuracy, 105 colonic tissue specimens from 59 patients. Although various research groups have shown the usefulness of Raman spectroscopy for distinguishing cancer tissues[7], the results of these independent studies are difficult to compare.

One reason for the difficulty is the interference of background fluorescence arising from endogenous fluorophores[12]. The excitation wavelength is chosen according to the type of tissue being examined, with the most common wavelengths being 532, 785, and 1064 nm. Most reports of the diagnostic potential of Raman spectroscopy for colorectal cancer were based on the use of an excitation wavelength of 785 nm[13], and a low signal-to-noise ratio and high level of background fluorescence often complicated the collection of spectral data. Excitation at a longer wavelength of 1064 nm is frequently used for the measurement of Raman signals in fresh tissues because of the relatively low background fluorescence and absence of photodamage[13]. However, it is difficult to acquire the weaker spectral signals generated by excitation at 1064 nm.

Furthermore, setting up for Raman spectroscopy is a technically detailed process. The laser beam, optical filter, spectrometer, microscope, and personal computer (PC) must all be calibrated for the detection of Raman spectra. In addition, the quality of spectroscopy has varied from institution to institution; there have been no standard devices and no standard methods for spectroscopic measurement. Thus far, the clinical application of Raman spectroscopy has been technically challenging despite its advantages.

Raman spectra ranging from 800 to 1800 cm^{-1} have been reported to efficiently distinguish normal and cancer tissues[9,10,12]. The individual Raman intensities, which are derived from the molecular structure of the sample, allow for tissue identification through the characterization of the tissue and cell components, such as the protein, DNA, and lipid contents. However, the technicalities involved make Raman spectroscopy difficult to apply clinically, the spectral range is still too broad for rapid analysis, and little is known about the spectra in detail, which is essential for clinical application.

We conducted a study in which we tested the feasibility of using an all-in one, small, handheld Raman spectrometer to facilitate tumor diagnosis. The device does not require a complicated setup, and the detected Raman spectra are immediately recorded by the device itself. This commercially available, portable spectrometer meets certain specifications and thus makes it possible to produce high-quality data and obtain highly reproducible measurements. In the study described herein, we used this spectrometer for the clinical analysis of colorectal cancer tissues, and with the goal of developing a new modality for the diagnosis of colorectal cancer, we further investigated which spectral range between 800 and 1800 cm^{-1} would best discriminate the tissues.

Methods

Patients and sample collection

The study involved 20 patients who underwent primary surgery for colorectal cancer at Showa University Fujigaoka Hospital between October 2016 and July 2018. All provided written, informed consent for their inclusion. From this group of patients, we obtained 46 tissues (25 normal and 21 cancer tissues) that were then prepared as 1-cm square-block samples (1-3 blocks per patient) and wrapped in a polyvinylidene chloride film. The study and all procedures involved were conducted under approval granted by the institutional review board of Showa University (IRB number: 2016012).

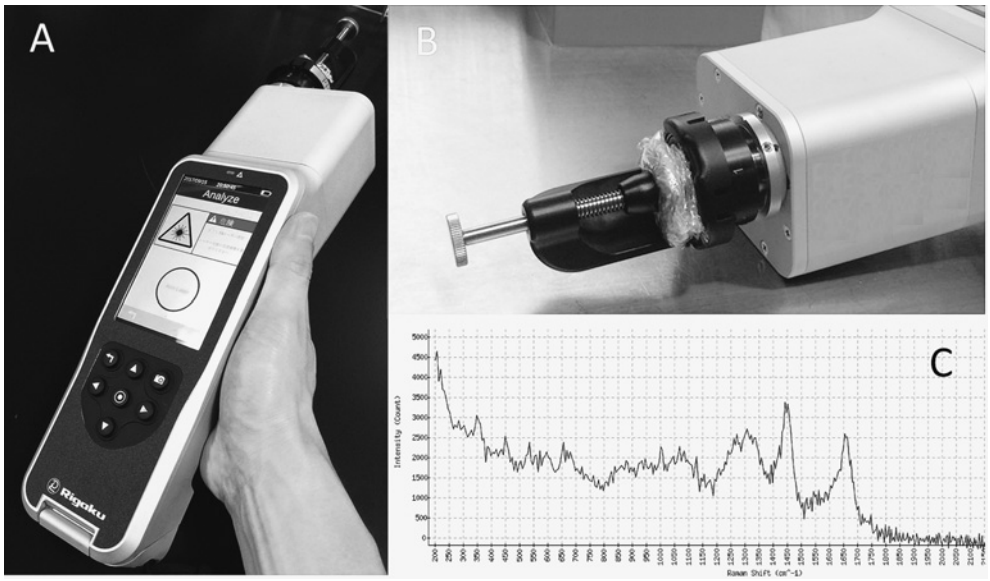

Figure 1. All-in-one Raman spectroscopy was performed using (A) a handheld Progeny™ spectrometer (Rigaku Raman Technologies Inc., Tokyo, Japan). (B) The tissue sample is placed in the holder attached to the front of the device and scanned. (C) A recorded Raman spectra.

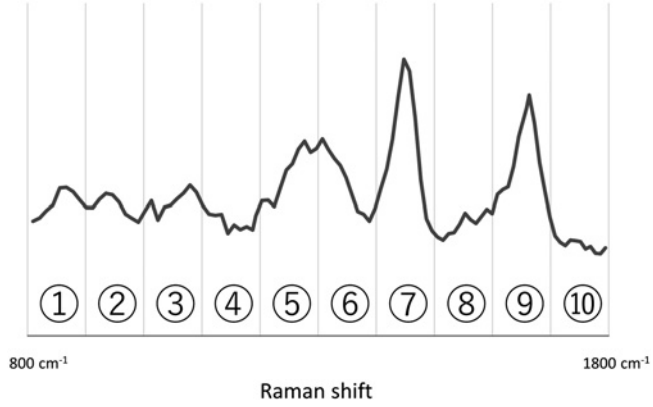

Figure 2. The Raman spectra ranging from 800 to 1800 cm^{-1} are divided into 10 regions of 100 cm^{-1} each.

The Raman spectrometer

We analyzed the tissue samples with the use of a Progeny spectrometer (Rigaku Raman Technologies Inc., Tokyo, Japan). This spectrometer is an all-in-one instrument equipped with a 1064-nm laser excitation source, an InGaAs detector, and a spectra recorder. It is only 29 cm in its longest dimension, and it weighs only 1.6 kg. Before using the device, we calibrated it per the manufacturer's instructions against the appropriate standard reference material. In actual use, a tissue block was placed in the holder attached to the front of the device. The tissue was irradiated at 3-5 random spots on the mucosal side at an average power of 490 mW, exposure time of 5000 ms, and average frequency of 30. The Raman spectra were scanned and recorded by the device and then downloaded to a PC for off-line analysis. A total of 168 Raman spectra (88 of normal tissues and 80 of cancer tissues) were analyzed in the spectral region of 800 to 1800 cm^{-1} (Figure 1).

Data analysis

The spectra obtained were analyzed in two steps. One hundred ninety-seven shifts were observed in the spectral range of 800 to 1800 cm^{-1}. To find the characteristic Raman shifts, the intensity of scattered light at each shift was evaluated. Intensity distribution was compared between normal tissues and cancer tissues, and differences were analyzed by Kruskal-Wallis test, with $p < 0.05$ considered statistically significant. The discriminatory power of the various intensity values was tested by means of nonparametric receiver operating characteristic (ROC) analysis. The area under the ROC curve (AUC), 95% confidence interval (95% CI), and sensitivity and specificity were calculated for various cut-off points so that the optimum cut-off point could be determined. These statistical analyses were performed with JMP ver.14 (SAS Institute Inc., Cary, NC, USA).

Principal component analysis (PCA) was then applied to the Raman spectra. PCA is a mathematical procedure that can be used to reduce a large set of variables to a small set that contains most of the information contained in the large set. Raman spectra ranging from 800 to 1800 cm^{-1} were divided into 10 regions of 100 cm^{-1} each (Figure 2). Thirteen kinds of principal components for 168 spectra were calculated in each of the 10 regions with the use of SYSTAT ver. 13 (Systat Software Inc., San Jose, CA, USA). Discriminant analysis of these principal components was used to deter-

Table 1. Patient and Tumor Characteristics (n = 20).

Age	73.5 (56-81) years
Sex	
Male	14 (70.0%)
Female	6 (30.0%)
Tumor location	
Right colon	6 (30.0%)
Left colon	14 (70.0%)
Tumor stage	
pT1	2 (10.0%)
pT2	1 (5.0%)
pT3	13 (65.0%)
pT4	4 (20.0%)
Tumor morphology	
Early colorectal cancer[a]	
Type I	0 (0.0%)
Type II	2 (10.0%)
Advanced colorectal cancer[b]	
Type I	1 (5.0%)
Type II	14 (70.0%)
Type III	2 (10.0%)
Type IV	0 (0.0%)
Type V	1 (5.0%)

Median (range) values or number (percentage) of patients are shown.
[a]According to the Paris-Japanese classification system (14).
[b]According to the Bormann classification system (14).

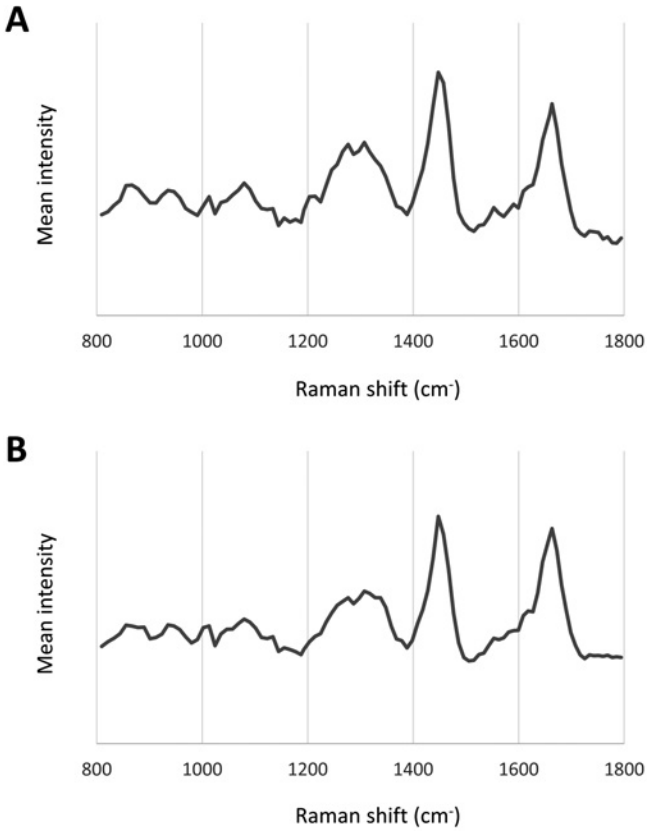

Figure 3. Mean intensity of Raman spectra from (A) normal colon tissues (n = 88) and (B) colorectal cancer tissues (n = 80).

mine the points that would best discriminate the groups based on the weights of the principal components in a sample. Individual principal components and various combinations of components from the 10 different regions were used for the discriminant analysis, and the sensitivity, specificity, and accuracy of the components were calculated to determine whether the cancer tissue could be identified by means of Raman spectroscopy. SYSTAT ver. 13 was also used for these analyses.

Results

Patient and tumor characteristics

The 20 patients who provided samples for the study were 14 men and 6 women who ranged in age from 56 to 81 years (the median age was 73.5 years). The clinical characteristics of these patients are shown in Table 1. The tumor was located in the left colon in 70% of the patients. The pathological stage was pT3 or pT4 in 85% of cases. Of the total 20 tumors, 90% were advanced colorectal cancers according to the Paris-Japanese classification, and 70% were Type II cancers according to the Borrmann classification[14].

Analysis of the Raman spectra

Similarity was found between normal tissues and cancer tissues in the shape of the spectrum and in the trend in the shift of the average spectrum of the Raman shift (from 800 to 1800 cm^{-1}; Figure 3). However, when the spatial distribution of the spectral intensity of the normal tissues was compared with that of cancer tissues, some statistically significant characteristic Raman shifts were found. The intensities at 1261 and 1427 cm^{-1} differed significantly between normal tissues and cancer tissues, and the intensities at 1276 and 1442 cm^{-1} differed slightly (Table 2). Because these differences were thought to reflect the differences in the biological composition and molecular structure of normal tissues vs. cancer tissues, ROC curves were drawn, and the AUCs were used for discriminant analysis. According to the values obtained (maximum AUC = 0.593 [95% CI = 0.501-0.678) for 1261 cm^{-1}, sensitivity = 49.3%, and specificity = 64.2%]; Table 2), these intensities at the Raman shift could not distinguish tumor tissues from normal tissues.

The results of the discriminant analyses are shown in Table 3. None of the 100 cm^{-1} regions with its principal components could reliably distinguish cancer tissues from normal tissues, accuracy ranged from 63.8% to 78.2%. The principal component that included all 10 spectral regions, from 800 to 1800 cm^{-1} (① ~ ⑩), provided the best discrimination, with 85.9% sensitivity, 82.3% specificity, and 84.1% diagnostic accuracy. When we tried various combina-

Table 2. Characteristic Raman Shifts that Differed between Normal and Cancer Tissues.

Raman shift	P value	AUC	95% CI	Sensitivity	Specificity
1261 cm^{-1}	$P < 0.05$	0.593	0.501-0.678	49.3%	64.2%
1427 cm^{-1}	$P < 0.05$	0.589	0.495-0.674	44.0%	60.5%
1276 cm^{-1}	$P = 0.143$	0.567	0.476-0.655	37.3%	65.4%
1442 cm^{-1}	$P = 0.132$	0.570	0.478-0.656	40.0%	61.7%

AUC: The area under the ROC curve, 95% CI: 95% confidence interval.

Table 3. Results of the Discriminant Analysis per Region and Combinations of Regions.

Region	Accuracy	Combined regions	Accuracy
① 800 cm^{-1} ~ 900 cm^{-1}	70.3%	① ~ ⑩	84.1%
② 900 cm^{-1} ~ 1000 cm^{-1}	71.2%	① ~ ⑤	78.1%
③ 1000 cm^{-1} ~ 1100 cm^{-1}	74.2%	⑥ ~ ⑩	76.5%
④ 1100 cm^{-1} ~ 1200 cm^{-1}	63.8%	① ~ ③	75.4%
⑤ 1200 cm^{-1} ~ 1300 cm^{-1}	70.6%	④ ~ ⑦	80.1%
⑥ 1300 cm^{-1} ~ 1400 cm^{-1}	78.2%	⑧ ~ ⑩	71.3%
⑦ 1400 cm^{-1} ~ 1500 cm^{-1}	65.6%	② ③ ⑥	83.4%
⑧ 1500 cm^{-1} ~ 1600 cm^{-1}	67.1%		
⑨ 1600 cm^{-1} ~ 1700 cm^{-1}	69.7%		
⑩ 1700 cm^{-1} ~ 1800 cm^{-1}	70.2%		

tions of the 10 spectral regions, the spectra from 1100 to 1400 cm^{-1} (④ ~ ⑦) provided good discrimination; accuracy was 80.1%. None of the 10 regions alone showed good discrimination. However, the results of the discriminant analysis of the 900 to 1000 cm^{-1} region, 1000 to 1100 cm^{-1} region, and 1300 to 1400 cm^{-1} region (②, ③, and ⑥, respectively) were relatively good. These three areas were selected as effective parameters, with 86.3% sensitivity and 80.7% specificity, and the accuracy was 83.4% for the discrimination of cancer using principal components when the analysis included all three regions (②, ③, ⑥). Calculating principal components on a PC can take a very long time if the area is broad and the dataset is huge, but with a limited area, results can be obtained quite rapidly. The time required for calculating the principal components in the three areas in this study was about half that required for all 10 regions from 800 to 1800 cm^{-1}.

Discussion

Raman spectroscopy was originally applied as a noninvasive analytical tool in the field of chemistry. Application has recently expanded to the fields of cell biology, biochemistry, and oncologic diagnostics. Raman spectroscopy has recently been shown to be useful for the detection of various cancers, and the possibility of discrimination between normal tissues and cancer tissues based on the characteristics of the spectra has been reported[6,7]. In this study, we examined the usefulness of an all-in-one, small, handheld Raman spectrometer with 1064-nm excitation in the diagnosis of colorectal cancer. We did this with a view toward the establishment of a spectroscopy technique that can be used globally for real-time in vivo tissue characterization. Furthermore, we searched for the specific region in the Raman spectra that would yield rapid and precise diagnosis. Because excitation at a wavelength of 1064 nm produces low background fluorescence, we expected to find a specific Raman shift to distinguish cancer tissues from normal tissues. The characteristic Raman shifts at 1261 and 1427 cm^{-1} differed significantly between these two types of tissue.

Raman spectra can reveal such secondary protein structures as the alpha helices and beta pleated sheets from a specific region—the so-called amide group. This group includes important structural information at three areas. Amide I band exists from 1600 to 1700 cm^{-1} and is derived from coupling between individual vibrations (C=O) in the alpha helix or beta sheet, which seems to be a characteristic of normal tissues[15]. Amide II band is derived from N-H bending and C-N stretching vibration in parallel or antiparallel beta sheets from 1500 to 1560 cm^{-1}. The Amide III band from 1220 to 1280 cm^{-1} is based on N-H in-plane bending vibration and frequently reported as a diagnostic region in some cancer tissues. Amide III is thought to be related to the angiogenesis process and presence of DNA and noncollagenous proteins[16]. The Raman shifts at 1261 and 1276 cm^{-1}, where we found differences in this study, are located within the Amide III range. A difference associated with a disorder in the secondary structure of the protein components of cancer tissues has often been observed in this range[16]. Our study data are consistent with such association

and appear to reflect biological changes characteristic of malignancy, such as increased protein and angiogenesis.

The spectra of the tissue samples in the 800 to 1500 cm^{-1} region provide rich biochemical information and are known as the fingerprint region[17]. Many Raman spectroscopy studies have focused on the fingerprint region for the analysis and diagnosis of cancer tissues[17]. Raman shifts at 1427 and 1442 cm^{-1} are part of the fingerprint region and are associated with CH_2 and CH_3 bending because of the methyl bonds in nucleic acids, proteins, lipid, and collagen. Rehman et al. reported increased nucleic acid and protein contents in cancer tissues[18]. The differences at 1427 and 1442 cm^{-1} that we found probably reflect these cytological changes associated with cell proliferation in malignancies.

Differences at 1261 and 1427 cm^{-1} were significant and indicative of more protein and nucleic acid in cancer tissues than in normal tissues. These Raman shifts, however, did not efficiently discriminate the cancer tissues examined in our study.

Because it has been difficult to find a Raman shift that can effectively distinguish tumor tissues, Raman spectra were analyzed by means of PCA by Kawabata et al.[19], who extracted principal components from samples and applied these components for discriminant analysis. In our study, we were able to discriminate the cancer tissues based on the principal components in the Raman spectra from 800 to 1800 cm^{-1}. Thus, the all-in-one Raman spectrometer that we used, Progeny, was shown to discriminate between normal tissues and cancer tissues with high accuracy, and our results were consistent with previously reported results derived from a non-portable spectrometer[20].

To enhance accuracy and determine the essential areas of the detected Raman spectra, which we considered important for rapid diagnosis, we also analyzed the spectra after dividing the Raman spectra into 10 regions. Principal components in separate areas suggested key areas for discriminate analysis. It has been reported that the regions from 900 to 1000 cm^{-1} and from 1000 to 1100 cm^{-1} were assigned to the content of protein in the fingerprint region, and the region from 1300 to 1400 cm^{-1} was related to nucleic acids[16]. We picked up these regions and combined them. We could perform discriminant analysis with the same quality using one-third of combined area from 900 to 1000 cm^{-1}, from 1000 to 1100 cm^{-1}, and from 1300 to 1400 cm^{-1} by decreasing the analysis time by approximately 50%. These results will aid in the rapid and efficient diagnosis of colorectal cancer.

As noted above, there have been many reports on the usefulness of the Raman spectra for the diagnosis of various cancers, but the results from several independent studies had little in common, were not replicable, and were difficult to compare. The main reason for this is the difference in the Raman spectrometer devices used for measurement. Most of the spectrometers used in previously reported studies were original, and thus, the quality of the measurements was not officially verified. The all-in-one Raman spectrometer that we used, Progeny, not only is compact but also meets rigorous MIL-STD-810 G standards and IP-68 specifications[20]. This device is especially suitable for in vivo assessment because the 1064-nm excitation light has little effect on the tissue, which is an advantage in terms of practical clinical applicability. We think that the use of the same device by various groups at various institutes in future studies will allow for the accumulation of a large dataset, facilitate comparison of the results obtained, and thus pinpoint characteristic intensities.

In conclusion, we have demonstrated that all-in-one Raman spectroscopy can be used for the diagnosis of colorectal cancer. Furthermore, we were able to find specific areas of Raman spectra for discrimination. The goal is rapid, noninvasive diagnosis. The Raman spectrometer that we used provides several key advantages for clinical use, not only as an all-in-one, handheld, device but also because the data are of high quality and reproducible. We envision a future in which independent Raman spectroscopy profiles of various colorectal lesions and cancers are known and can be widely applied clinically for diagnosis. If this spectrometer were to be widely used at other institutes, we could collect data of the same high quality. This would make it easy to understand the meaning of the characteristic spectra and would encourage development of devices that could be used commonly for the diagnosis of colorectal cancer. Raman spectroscopy performed with a handheld device has potential to become a clinically powerful tool for improving the diagnosis of colorectal cancer.

Conflicts of Interest

There are no conflicts of interest.

Source of Funding

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