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Potential Application of Raman Spectroscopy for Real-time Diagnosis and Classification of Colorectal Cancer

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Abstract: Raman spectroscopy has become a remarkable tool for real-time detection and diagnosis of various cancers. We investigated whether Raman spectroscopy can be used to diagnose colorectal cancer. Samples of cancerous and non-cancerous tissues were obtained from 12 patients undergoing surgery for such cancer. A handheld Raman spectrometer employing an excitation wavelength of 1,064 nm was used at 94 points: 48 points on cancerous tissues and 46 points on normal tissues. Using principal component analysis (PCA), we selected 12 PCs from the Raman spectra obtained at each of the 94 observed points. We then used linear discriminant analysis (LDA) to distinguish cancer from normal tissues and early cancer from advanced cancer, and we calculated sensitivity, specificity, and accuracy of the Raman spectroscopy for such diagnoses. We immediately confirmed that there was no local temperature rise, color change, or damage at the irradiated points, and we found that Raman spectroscopy was able to distinguish cancer from normal tissues with a sensitivity of 87.5%, specificity of 82.6%, and accuracy of 85.1%. In addition, Raman spectroscopy distinguished early cancer from advanced cancer with a sensitivity of 85.7%, specificity of 83.3%, and accuracy of 85.4%. Thus, near-infrared Raman spectroscopy shows potential as an objective, rapid, non-invasive diagnostic modality for colorectal cancer.

Key words: colorectal cancer, Raman spectroscopy, handheld spectrometer, 1,064-nm excitation, diagnostic device

Introduction

Colorectal cancer is one of the most common cancers worldwide and, in 2016, it was the second most common cause of death from cancer in Japan[1)]. Five-year survival is 96.6% for patients with localized colorectal cancer, 72.1% for those with lymph node metastasis, and 15.8% for those with distant metastasis[2)]. Thus, early diagnosis and treatment greatly influence the prognosis. Currently, pathological analysis of biopsy specimens is regarded as the standard procedure for definitive diagnosis and staging of colorectal cancer; however, pathological examination is both time-consuming and costly.

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Raman spectroscopy is emerging as an important tool for real-time detection and diagnosis of various cancers. First described by C.V. Raman in 1928, Raman spectroscopy is an imaging technique based on inelastic scattering of monochromatic light from a laser, whereby photon frequencies change upon interaction with a sample[3–5]. When a molecule is excited by the photon, the frequency of the re-emitted photon shifts up or down relative to the original monochromatic frequency. This phenomenon is called the Raman shift (or Raman effect). The shift in frequency provides unique information on molecules[6, 7] that allows for tissue fingerprinting by characterizing the protein, DNA, and lipid content of cells. Thus far, clinical application of Raman spectroscopy has been difficult due to masking of the relatively weak Raman scattering by the strong fluorescence emitted by biological tissue. Indeed, some human tissue samples emit fluorescence even when excited at 800 nm[8, 9]. Long-wavelength, near-infrared light has been shown to prevent interference by fluorescence[10]. Additionally, use of near-infrared light is advantageous for spectroscopic characterization and analysis of biological tissues because the samples are not damaged by the laser beam-generated heat[11]. We therefore investigated whether Raman spectroscopy, performed with a handheld spectrometer, has the potential for practical, objective, rapid diagnosis of surgically resected colorectal cancer.

Materials and methods

Tissue samples

The specimens of adenocarcinoma and adjacent normal tissues analyzed in this study came from 12 patients undergoing surgical resection for pathologically diagnosed colorectal cancer at the Department of Gastroenterological and General Surgery, Showa University Fujigaoka Hospital, from October 2016 to March 2017. The excised tissues were cut into 1-cm square blocks, wrapped in plastic, and stored up to one hour before examination.

Patient and tumor characteristics are summarized in Table 1. Located in the ascending colon (n＝3, 25.0%), transverse colon (n＝1, 8.3%), sigmoid colon (n＝2, 16.7%), or rectum (n＝6, 50.0%), the cancerous tissues were classified histologically as well-differentiated (n＝6, 50%) or moderately differentiated adenocarcinoma (n＝6, 50%), and morphologically as type 0 (n＝2, 16.7%), type 2 (n＝7, 58.3%), type 3 (n＝2, 16.7%), type 4 (n＝0, 0.0%), or type 5 (n＝1, 8.3%); there were no type 1 tumors. Two of the cancers (16.7%) were early cancers (submucosal invasion), and 10 (83.3%) were advanced cancers (muscularis propria invasion [n＝1], subserosal penetration [n＝5], and serosal penetration [n＝4]). Tumor stages according to the UICC (International Union Against Cancer) were as follows: stage I (n＝2, 16.7%), stage IIA (n＝2, 16.7%), stage IIB (n＝1, 8.3%), stage IIIA (n＝1, 8.3%), stage IIIB (n＝3, 25.0%), stage IVA (n＝2, 16.7%), and stage IVB (n＝1, 8.3%).

Ethics statement

The Institutional Review Board of Fujigaoka Hospital approved the study protocol (permission number: 2016012). All 12 patients were informed of the study and provided written consent for scientific use of the surgical specimens.

Table 1. Patients and Tumor Characteristics

Patient	Age (years)	Sex	Tumor location	Histological type	Morphological type	T	N	M	Stage	Tumor progression
1	76	M	T	tub1	2	3	1a	0	ⅢB	Advanced
2	56	M	S	tub2	3	4a	X	1b	ⅣB	Advanced
3	69	M	RS	tub2	2	3	0	0	ⅡA	Advanced
4	81	F	Ra	tub1	0-Ⅱa	1	0	0	Ⅰ	Early
5	73	M	RS	tub2	5	3	1b	0	ⅢB	Advanced
6	73	M	RS	tub1	2	4a	0	0	ⅡB	Advanced
7	73	M	A	tub1	2	3	0	0	ⅡA	Advanced
8	74	F	A	tub2	0-Ⅱa	1	0	0	Ⅰ	Early
9	76	M	A	tub1	2	4a	1b	0	ⅢB	Advanced
10	67	M	Rb	tub2	2	2	1b	0	ⅢA	Advanced
11	81	M	S	tub1	3	3	2a	1a	ⅣA	Advanced
12	68	M	RS	tub2	2	4a	1a	1a	ⅣA	Advanced

A : ascending colon, T : transverse colon, S : sigmoid colon, RS : rectosigmoid
Ra : rectum above peritoneal reflection, Rb : rectum below peritoneal reflection
tub1 : well differentiated adenocarcinoma, tub2 : moderately differentiated adenocarcinoma

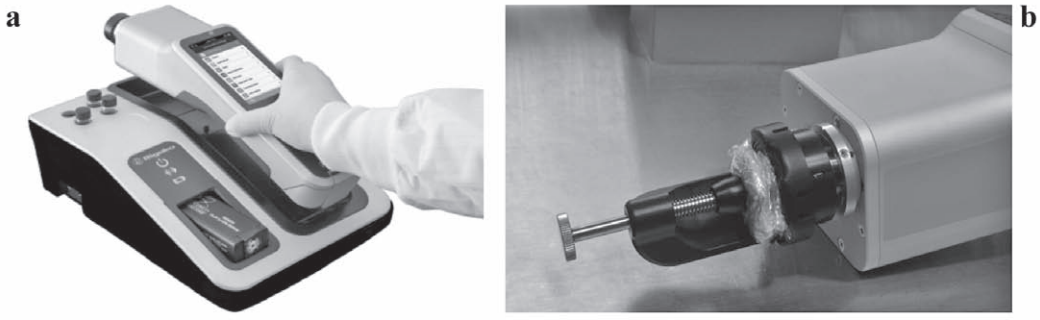

Fig. 1. (a) The handheld Raman spectrometer, Progeny™ (Rigaku Raman Technologies Inc.) equipped with a 1,064-nm laser, (b) The handheld Raman spectrometer in use.

Handheld Raman spectrometer

We used an easy-to-handle Progeny™ spectrometer (Rigaku Raman Technologies Inc., Tokyo, Japan) [12, 13], measuring 29.9 × 8.0 × 7.4 cm, weighing 1.6 kg, and equipped with a thermoelectrically cooled InGaAs detector that is highly sensitive to near-infrared light. Radiation can be measured correctly, even through vinyl, by adjustment of the focal length in increments of 0.5 mm. The Progeny™ spectrometer overcomes the interference of sample-induced fluorescence by virtue of a unique 1,064-nm excitation laser for expanded positive material identification.

Irradiation and Raman spectra measurement

Spectroscopic analysis of the tissue samples was performed at room temperature. Raman spectra of the colorectal cancer tissues (1-3 blocks per patient) and adjacent normal tissues (1-3 blocks per patient) were generated with a 1,064-nm excitation laser (Fig. 1a). The tissue was

irradiated on the mucosal side through the plastic wrap (Fig. 1b), with each block irradiated at 3-5 spots randomly and checked for any laser damage. The settings were as follows: average power, 490 mW; exposure time, 5,000 ms; averaging frequency, 30 count. The InGaAs detector allowed both measurement of Raman scattering and analysis of Raman spectra, the latter of which were expressed in wavenumbers ranging from 800 cm^{-1} to 1,800 cm^{-1}. Comparisons were then made between the spectra of the cancer tissues and those of the normal tissues.

Statistical analysis

Spectral data were processed and analyzed statistically by means of SYSTAT ver. 13 (Systat Software Inc., San Jose, CA, USA). We used principal component analysis (PCA) to identify and extract the major trends within a given spectral data set. PCA produces principal components (PCs) to concentrate all spectral information with minimal information loss[14]. The first PC accounts for the maximum amount of variance present in the spectral data set, whereas successive PCs account for features contributing to progressively smaller variances. We selected 12 PCs from the Raman spectra collected at each of the 94 observed points (Fig. 2). No single PC or combination of PCs could efficiently distinguish cancer tissues and normal tissues. Therefore, we used all 12 PCs to find the best discriminating components. This is in keeping with Kawabata *et al*[15], who used all the PCs (10 in their case) to develop a Raman spectroscopy-based system for ex vivo diagnosis of gastric cancer. As a second step, the 12 PC were entered

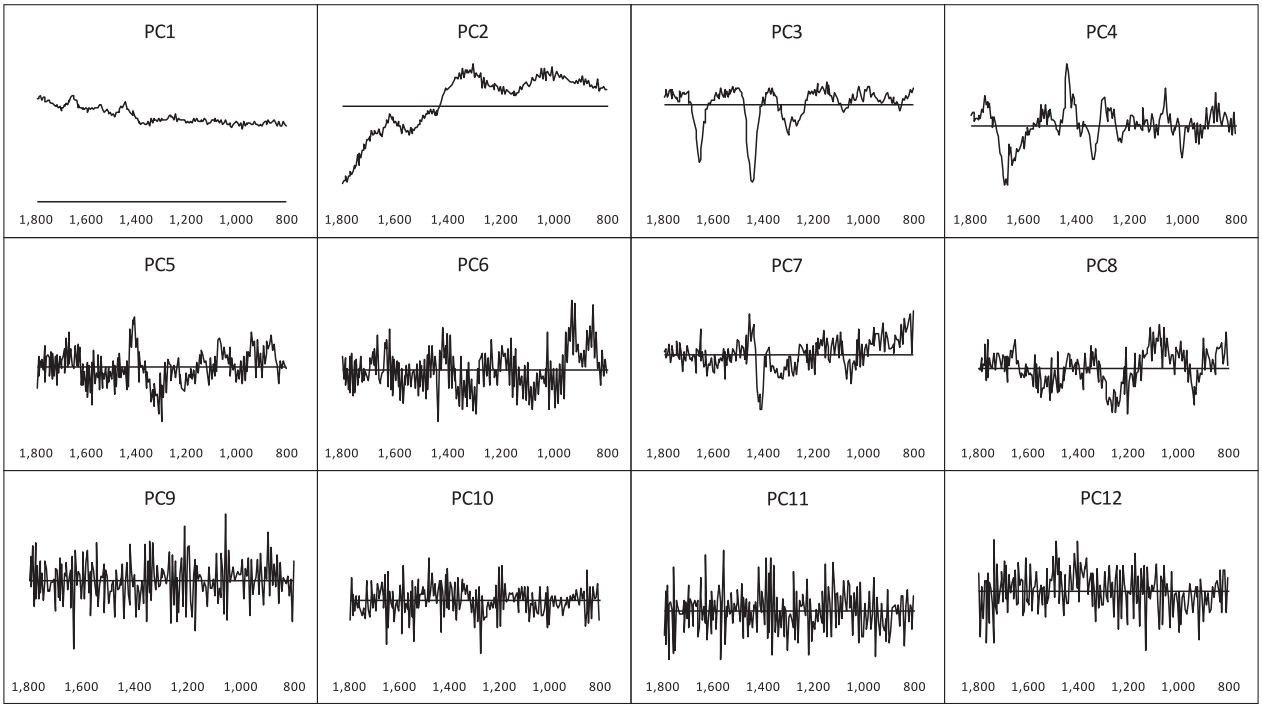

Fig. 2. The 12 principal components (PCs) obtained from analysis of the 94 Raman spectra

into a linear discriminant analysis (LDA), to distinguish cancer tissues from normal tissues and early cancer from advanced cancer. We calculated the sensitivity, specificity, and accuracy of Raman spectroscopy for such diagnoses.

Results

Investigation of Raman spectra

Across the 24 irradiated tissues, we detected no local temperature rise, color change, or damage at any of the 94 points. The general pattern similarity amongst all 94 Raman spectra obtained from the cancerous and normal tissue specimens is shown in Figure 3a, with the average spectra for both tissue types shown in Figure 3b. For each spectra, typical Raman peaks were identified at 1,658 cm^{-1}, 1,447 cm^{-1}, and 1,271 cm^{-1}, which correspond to the protein amide I band, the CH_2 bending, and the protein amide III band, respectively. As depicted in Figure 3c, the spectral differences (cancerous tissue spectra minus normal tissue spectra) revealed higher intensities for the cancer tissues in comparison to the normal tissues, especially at 1,663 cm^{-1} and 1,332 cm^{-1}; however, at 1,442 cm^{-1} the spectral intensities of the cancer tissues were lower than those of normal tissues.

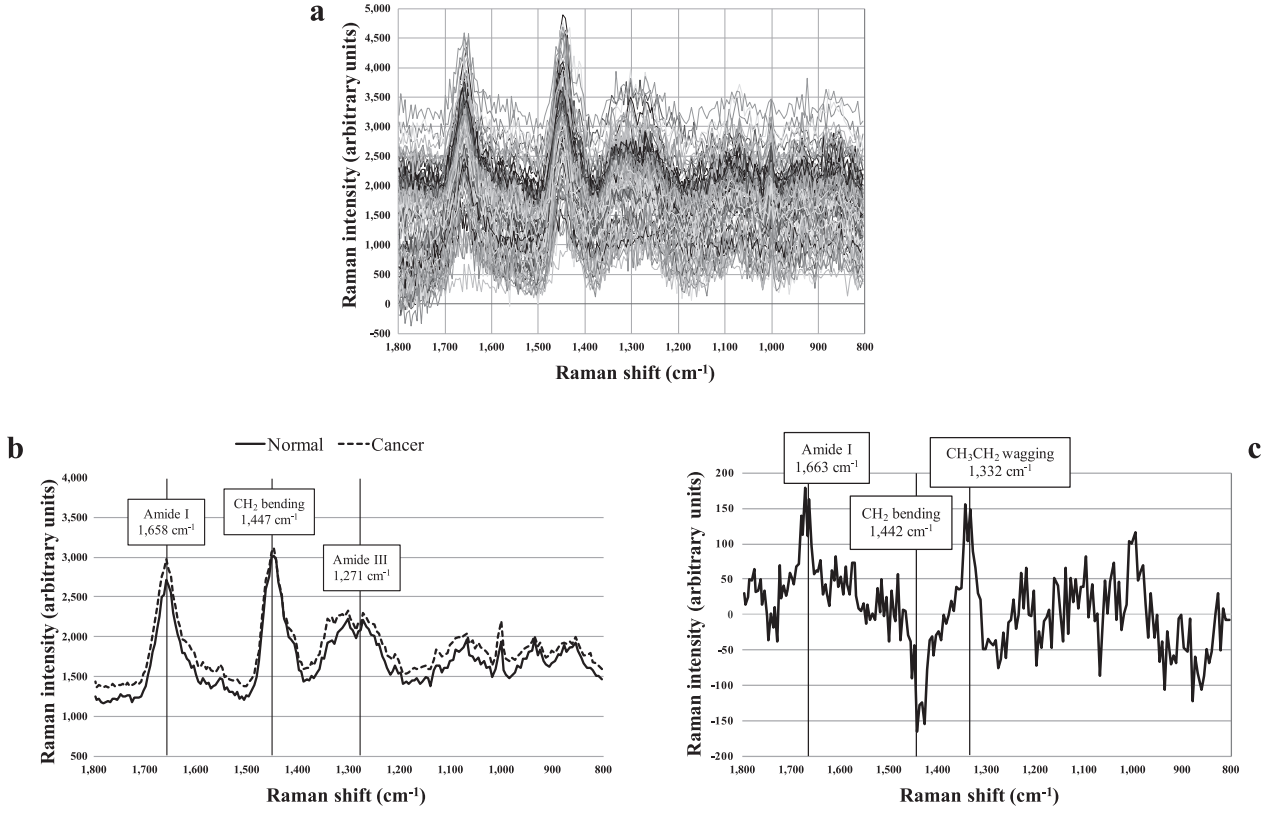

Fig. 3. (a) The total of 94 Raman spectra of cancer tissues and normal tissues, (b) Average Raman spectra of cancer tissues and normal tissues, (c) Differences in spectra (cancer tissues minus normal tissues).

PCA and LDA

PCA was used for all 94 Raman spectra to detect diagnostically important spectral differences. The 12 PCs obtained from the 94 Raman spectra (PC1-12) are shown in Figure 2. When all 12 PCs were analyzed using LDA, 42 of the 48 spectra from cancer tissues and 38 of the 46 spectra from normal tissues were accurately classified (Table 2), with a sensitivity of 87.5%, specificity of 82.6%, and accuracy of 85.1%. We also examined whether Raman spectroscopy could distinguish early cancer (T1) from advanced cancer (T2–T4) using the 48 cancerous tissue spectra. Then again using PCA and LDA, we determined that 5 of 6 spectra from the early cancers and 36 of 42 spectra from the advanced cancers were accurately classified (Table 3), with a sensitivity of 85.7%, specificity of 83.3%, and accuracy of 85.4%.

Discussion

Raman spectroscopy was first used in the field of chemistry and then later developed as a scientific tool for examining pathological tissues. The advantage of Raman spectroscopy is that it can be used to provide rapid, noninvasive detection. Raman spectroscopy has been used in the detection of various cancers including gastrointestinal[15–18], breast[19], lung[10, 20], uterine[21], nasopharyngeal[22], laryngeal[23], and skin[24] cancers. Peak positions and vibrational modes already assigned are shown in Table 4. In gastrointestinal tissue, the differences in Raman spectra have been recognized mainly at 1,655 / 59 cm^{-1}, 1,450 / 3 cm^{-1}, 1,310 cm^{-1}, 1,260 cm^{-1}, and 1,003 cm^{-1} [25]. In our colorectal tissue samples, Raman peaks were typically identified at 1,658 cm^{-1} (protein

Table 2. Discrimination analysis of cancer and normal tissue

		Pathological diagnosis	
	(n = 94)	Cancer	Normal
Raman classification	Cancer	42	8
	Normal	6	38
	Total	48	46

Sensitivity 87.5%, Specificity 82.6%, Accuracy 85.1%

Table 3. Discrimination analysis of advanced and early cancer

		Pathological diagnosis	
	(n = 48)	Advanced cancer	Early cancer
Raman classification	Advanced cancer	36	1
	Early cancer	6	5
	Total	42	6

Sensitivity 85.7%, Specificity 83.3%, Accuracy 85.4%

Table 4. Peak position and vibrational mode assignment

Peak position (cm^{-1})	Vibrational mode	Major assignments
≈ 1,000	Symmetric ring breathing	Phenylalanine
1,240–1,280	Amide III (C-N stretching)	Proteins (α-helix), lipids
1,300–1,315	CH_3CH_2 twisting	Nucleic acids, collagen, lipids
≈ 1,335	CH_3CH_2 wagging	Nucleic acids, collagen
≈ 1,450	CH_2 bending	Lipids, proteins
1,650–1,665	Amide I (C = O stretching)	Proteins (α-helix), lipids

amide I), 1,447 cm^{-1} (CH_2 bending), and 1,271 cm^{-1} (protein amide III), indicating that Progeny™ was able to measure Raman spectra precisely despite the emission of fluorescence from the irradiated tissues.

Min *et al*[10] reported that when lung cancer develops, the increased amount of protein makes the tissues dense, and it is the most likely cause of the increased amide I band intensity in cancer tissues. Furthermore, Feld *et al*[26] reported intense nucleic acid vibrational modes in adenocarcinoma, indicative of a higher nucleic acid content in the samples. In our study, the difference in spectra (cancer minus normal) was great at amide I (mainly protein) and in association with CH_3CH_2 wagging (mainly nucleic acid), supporting greater amounts of protein and nucleic acid in cancer tissues than in normal tissues. Feld *et al*[26] also reported more intense signals from lipids in their samples of normal tissue. The small difference in spectra (cancer minus normal) that we observed in association with CH_2 bending (lipid) is in agreement with their findings.

To detect statistically significant differences in spectra between cancer tissues and normal tissues, we used PCA and LDA, which are multivariate analysis techniques used in many investigations of Raman spectroscopy-based cancer diagnosis[14, 15, 17, 22, 23]. Our PCA and LDA results were consistent with the results of these previously reported studies in that Raman spectroscopy successfully and accurately discriminated between cancer tissues and normal tissues. In addition, we found that Raman spectroscopy could discriminate with high accuracy between early cancer and advanced cancer. In comparison to visible light, near-infrared light can penetrate more deeply into tissue, making Raman spectroscopy well suited to minimizing tissue fluorescence[7]. Thus, Raman spectroscopy may become useful for evaluating the depth of tumor invasion.

Raman spectroscopy is especially suitable for *in vivo* assessment because the excitation light, regardless of its power, does not affect the tissue[4]. We confirmed in our study that the tissues irradiated by Raman spectroscopy were not damaged, and thus we speculate that the 1,064-nm laser can be used safely *in vivo*, although further studies must be carried out to ensure that Raman scanning will not damage DNA in normal tissues.

In the study described herein, we focused on practical clinical applicability by using a handheld Raman spectrometer. Indeed, the portability of Progeny™ is often exploited in the aftermath of disasters. Miniaturizing a device often compromises its accuracy and performance, and thus large spectrometers are most often used for cancer detection. However, we were able to diagnose colorectal cancer with a handheld Raman spectrometer equipped with a high performance detector. Technological innovation has allowed for reduction in the size of Raman spectrometers, and investigators have recently reported using endoscopic Raman spectroscopy probes for real-time diagnosis and assistance in determining an adequate surgical margin during endoscopic resection[27, 28]. We anticipate that further development of intraoperative devices will aid in determining the excision line or extent of lymph node dissection when intraoperative assessment of colorectal tissues from the serosa side, lymph nodes, and nearby organs and structures (e.g., uterus, prostate, ureter, and blood vessels) is needed.

In conclusion, we investigated whether near-infrared Raman spectroscopy performed with a

handheld spectrometer with 1,064-nm excitation can be applied clinically for the diagnosis of colorectal cancer, and we obtained favorable results. We anticipate that similar applications of Raman spectroscopy will become increasingly important for both rapid and objective assessments of cancer and non-invasive procedures.

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Conflict of interest disclosure

The authors have no conflict of interest to declare.

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