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Suppressive effect of RAS inhibitor manumycin A on aberrant crypt foci formation in the azoxymethane-induced rat colorectal carcinogenesis model

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Abstract

Background and Aim: The chemopreventive effect of RAS inhibitors on colorectal cancer is unknown. Because aberrant crypt foci (ACF), earliest preneoplastic lesions, are highly positive for K-RAS mutation, RAS inhibitors are likely to be effective for chemoprevention. Therefore, in the present study, the suppressive effect of a RAS inhibitor, manumycin A, on ACF formation in an azoxymethane (AOM)-induced rat colorectal carcinogenesis model was investigated.

Methods: Rats injected with AOM were administered manumycin A (30 mg/kg) subcutaneously thrice weekly for 8 weeks or for 4 weeks (latter half), sacrificed at 8 weeks, and examined for ACF in the colorectum. Phosphorylated ERK and Ki-67 expression was evaluated by immunohistochemistry. Apoptosis was assessed by TUNEL staining.

Results: The mean number of ACF in the 8-week manumycin A group (72.9 ± 20.1) was significantly lower than in the vehicle group (155.6 ± 56.7, $P < 0.01$), and it was significantly lower even in the 4-week manumycin A group than in the vehicle group (92.2 ± 13.0 *vs* 222.3 ± 83.3, $P < 0.01$). The positive rate for phosphorylated ERK in the manumycin A group (13.5 ± 19.2%) was significantly lower than in the vehicle group (50.2 ± 19.8%, $P < 0.01$). The positive rate for Ki-67 in the manumycin A group (2.2 ± 3.4%) was significantly lower than in the vehicle group (14.7 ± 8.2%, $P < 0.01$). There were significantly more terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling-positive cells in tissue samples from the manumycin A group *versus* the vehicle group (8.6 ± 9.7% *vs* 2.9 ± 2.0%, $P < 0.05$).

Conclusion: Manumycin A suppressed ACF formation in the AOM-induced colorectal carcinogenesis model, demonstrating that RAS inhibitors may be very effective for chemoprevention of colorectal cancers.

Introduction

Colorectal cancer is the third most common cancer in men and the second most common form in women worldwide.[1] Chemoprevention is an important and effective strategy for reducing the incidence of colorectal cancer. Because it is well accepted that colorectal cancer develops through an adenoma-carcinoma sequence, chemoprevention trials targeting adenoma (polyps) have been performed using several candidate agents. It has been reported that aspirin or non-steroidal anti-inflammatory drugs such as sulindac inhibit the development of adenoma by 20–35%.[2,3] It has also been reported that cyclooxygenase-2 inhibitors inhibit the development of adenoma by 30–55%.[4,5] However, these drugs are associated with gastrointestinal and cardiovascular toxicity and it is therefore not possible to use them continuously for the prevention of colorectal cancer.[6]

It has been reported that K-RAS mutation plays an important role in the adenoma-carcinoma sequence of colorectal carcinogenesis. The positive rates of K-RAS mutations are reported to be 9–58% in adenoma and 47% in cancer.[8–11] Because K-RAS mutations are positive at codons other than the codon 12 and the direct sequencing method routinely employed lacks sufficient sensitivity, it has been suggested that these K-RAS mutation rates underestimate the true incidence of K-RAS mutation.

We have observed aberrant crypt foci (ACF), a minute type of colorectal lesion, using magnifying colonoscopy in normal subjects and patients with adenoma and cancer, suggesting that ACF are precursor lesions of the adenoma-carcinoma sequence.[12] We

and other investigators found that ACF are frequently positive for K-RAS mutation.[10,13] Furthermore, we found that K-RAS mutation induces overexpression of glutathione S-transferase-π (GST-π) via activator protein 1 activation and that GST-π plays a pivotal role in the resistance of apoptosis to bile acids in the colorectum.[14] Therefore, it appears that K-RAS, which exhibits mutations in ACF, the earliest lesion associated with colorectal carcinogenesis, is the most suitable target molecule for chemoprevention.

Manumycin A is a natural product derived from *Streptomyces parvulus* that acts as a potent and selective RAS farnesyltransferase inhibitor.[15] The enzyme farnesyltransferase modifies RAS and other proteins with the farnesyl isoprenoid lipid that is required for their correct cellular localization and biological activity.[16] Recently, the antineoplastic activity of manumycin A has been demonstrated in various experimental systems.[17–20] It has been reported that manumycin A inhibits cell growth of tumor cells by suppression of RAS farnesylation.[21] It has also been reported to induce apoptosis in various cancer cell lines including human colon tumor cells.[22,23] Furthermore, manumycin A induces the death of breast cancer cells via cytoplasmic vacuolation.[24] However, the chemopreventive effect of manumycin A on colorectal cancer has not been studied to date, nor has the chemopreventive effect of RAS inhibitors, such as FTI276 and L744,832, on colorectal cancer been investigated. Therefore, in the present study, we investigated the inhibitory effect of the RAS inhibitor manumycin A in a rat model of azoxymethane (AOM)-induced colorectal carcinogenesis, which mimics human colorectal carcinogenesis with high K-RAS mutation in ACF, as well as adenoma and cancer.

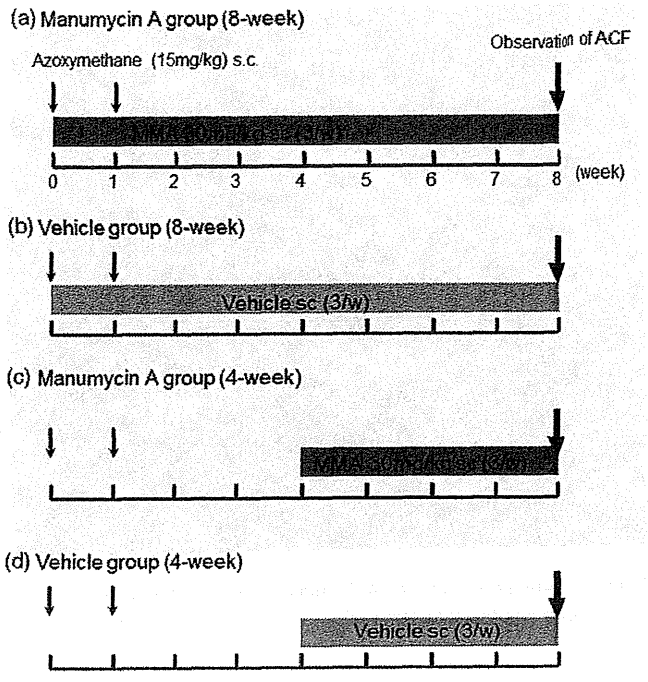


Figure 1 Experimental protocol. Five-week old rats were administered azoxymethane at a dose of 15 mg/kg (once a week for 2 weeks). (a) Manumycin A (MMA) was administered to rats at a dose of 30 mg/kg (three times per week) for 8 weeks. (b) Only vehicle was administered to rats for 8 weeks. (c) Manumycin A was administered to rats at a dose of 30 mg/kg (three times per week) only during the latter 4 weeks of the study (weeks 5–8). (d) Only vehicle was administered to rats for the latter 4 weeks of the study (weeks 5–8). ACF, aberrant crypt foci.

Materials and methods

Animals and treatment protocol. All animal experiments were approved by the University of Tokushima Graduate School. Male F344 rats (age 5 weeks) were purchased from Charles River Japan, Inc. (Tokyo, Japan) and maintained in the animal housing facility at the University of Tokushima Graduate School. Manumycin A was obtained from Enzo Life Sciences (Tokyo, Japan). AOM was purchased from Sigma-Aldrich Co. (St Louis, MO). Animals were maintained on a 12-h light/dark cycle with free access to water and food. The experimental protocol for the present study is shown in Figure 1. AOM was administered to 28 rats subcutaneously at a dose of 15 mg/kg once a week for 2 weeks, and the animals were assigned to four groups (Fig. 1). Manumycin A was dissolved in dimethyl sulfoxide (DMSO), further diluted in phosphate buffered saline (PBS), and administered subcutaneously to rats at dose of 30 mg/kg thrice weekly for 8 weeks or for the latter 4 weeks of the study, according to the method of Jamroz-Wiśniewska *et al*.[25] Control rats received vehicle only for either 8 weeks or for the latter 4 weeks of the study.

ACF examination. Entire colorectums were carefully removed, immersed in 10% neutral buffered formalin, opened longitudinally from the cecum to the anus, and placed between filter paper. Subsequently, the tissues were fixed in 10% neutral buffered formalin for 24 h. They were then stained with 0.2% methylene blue in saline and placed, mucosal side up, on microscope slides; ACF was then examined under a stereoscopic microscope. Lastly, the colorectal mucosa including ACF was excised and paraffin-embedded to prepare cross sections (4 μm thickness) for hematoxylin and eosin staining and for immunohistochemistry.

Immunohistochemistry. Immunohistochemical staining was performed using the streptavidin-biotin peroxidase method with labeled streptavidin-biotin (Dako, Tokyo, Japan), as we described previously.[26] Briefly, formalin-fixed paraffin-embedded sections were cut 3 μm thick, deparaffinized in xylene, and then rehydrated in graded ethanol solutions and PBS. The slides were autoclaved for 15 min at 121°C in target retrieval solution (pH 9.0) (Dako). They were then blocked with protein block (Dako) and incubated with rabbit antihuman Ki-67 polyclonal antibody or mouse antihuman phosphorylated ERK (p-ERK) monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as the primary antibody. After washing with PBS, the slides were incubated with biotinylated secondary antibody followed by incubation with horseradish-streptavidin and visualization with 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako). Finally, the sections were counterstained with Mayer's hematoxylin.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining. An Apoptosis in situ Detection Kit (Wako, Osaka, Japan) was used to label apoptosis-induced DNA strand breaks by

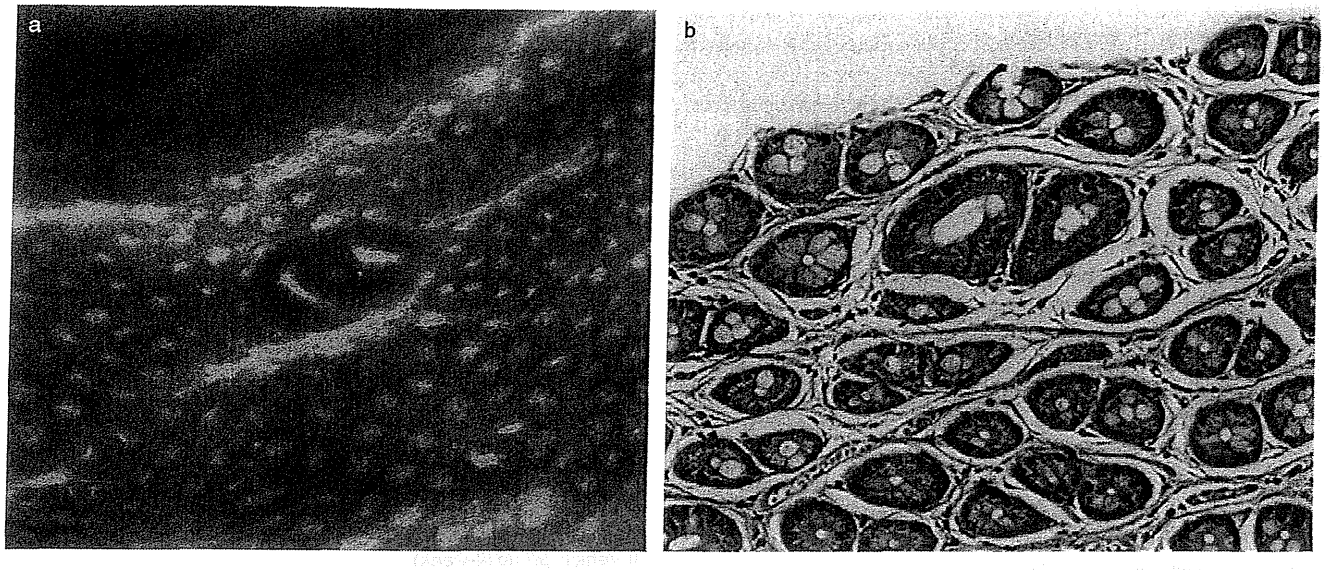


Figure 2 Aberrant crypt foci (ACF) induced by azoxymethane. (a) ACF observed under a light microscope with methylene blue staining, consisting of two large crypts; (b) histological findings of the corresponding ACF.

the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) technique. In brief, formalin-fixed paraffin-embedded sections of rat colorectal mucosa were placed on silanized slides (Dako). After deparaffinization, they were immersed in a protein-digestion enzyme solution at room temperature for 5 min and incubated for 10 min at 37°C in a terminal deoxynucleotidyl transferase substrate solution. Subsequently, the sections were immersed in 3% hydrogen peroxide to block endogenous peroxidase activity and then incubated with peroxidase-conjugated antibody at 37°C for 10 min. The DAB was added, and counterstaining was performed with methyl green.

Statistics. The number of ACF was compared between the manumycin A and vehicle groups by Student's *t*-test. Positivity rates for p-ERK and Ki-67 immunostaining and TUNEL staining were also compared between the manumycin A and vehicle groups by Student's *t*-test.

Results

General observation. Rats administered manumycin A appeared to be healthy throughout the experiment, and no apparent signs of toxicity were observed. There were no appreciable changes in bodyweight gain or diet intake between the manumycin A and vehicle groups (data not shown). There were no significant differences in the weights of liver, kidney, lung, or spleen between the two groups.

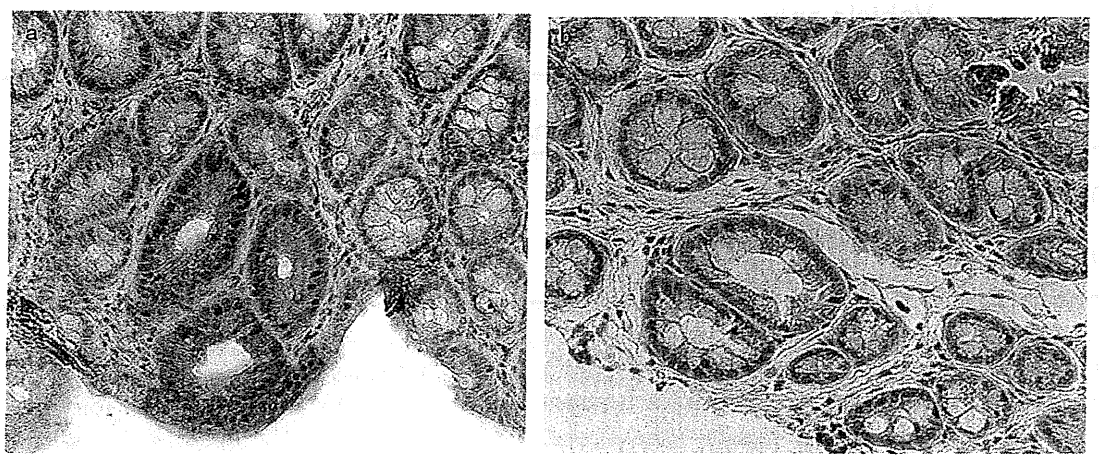
Manumycin A reduced the number of ACF in AOM-injected rats. In the first setting, manumycin A was administered for 8 weeks from the first day of AOM administration, and the rats were sacrificed at 8 weeks for observation of ACF (Fig. 1a,b). ACF in methylene blue-stained colorectum were identified and counted under a stereoscopic microscope. Figure 2a shows a typical ACF consisting of two large crypts densely stained with methylene blue. Figure 2b shows the histological findings of the corresponding ACF, which consisted of crypts twofold to threefold larger than the surrounding normal crypts. Nuclear disorientation, irregular arrangement, and chromatin condensation were observed in the epithelial cells in the aberrant crypts.

The mean number of ACF in the manumycin A group was 72.9 ± 20.1, which was significantly smaller than in the vehicle group (155.6 ± 56.7) ($P = 0.003$). When the number of ACF was assessed according to the size of the ACF (1, 2, 3, 4 crypts or 5 crypts≦), a significant difference was observed between the manumycin A and vehicle groups for all sizes of ACF. However, the inhibitory effect was more pronounced for larger ACF (Table 1). In the second setting, manumycin A was administered for 4 weeks, from week 5 to week 8, and the suppressive effect on ACF formation was evaluated. The mean number of ACF in the manumycin A group was 92.2 ± 13.0, which was significantly lower than in the vehicle group (222.3 ± 83.3) ($P = 0.004$). When the number of ACF was assessed in terms of the size of ACF, the suppressive effect on ACF formation was more evident for larger sized ACF.

Manumycin A inhibited ERK phosphorylation and Ki-67 positivity in ACF. Manumycin A, a RAS farnesyltransferase inhibitor, is thought to act by inhibiting signal transduction via the RAS/RAF/MEK/ERK pathway and to suppress cell proliferation. To clarify the mechanism of action, AOM-injected rats received manumycin A at dose of 30 mg/kg four times beginning at 5 weeks. They were then sacrificed at 6 weeks, and the ACF tissues were immunohistochemically examined for p-ERK and Ki-67 expression. Figure 3 shows representative results of immunostaining for p-ERK. The apparent expression of p-ERK in the cytoplasm and nucleus of ACF cells was detected in

Table 1 Effect of manumycin A on number of aberrant crypt foci (ACF) in rats induced by azoxymethane

	Total No. of ACF	No. of ACF				
		1 crypt	2 crypts	3 crypts	4 crypts	5 crypts ≤
8-week treatment						
Vehicle	155.6 ± 56.7	35.4 ± 16.2	51.8 ± 23.4	36.1 ± 16.9	19.8 ± 12.8	12.6 ± 7.1
Manumycin A	72.9 ± 20.1	35.4 ± 16.2	25.1 ± 11.2	15.0 ± 9.8	7.6 ± 2.7	4.1 ± 2.0
P value	0.003	0.018	0.015	0.015	0.006	0.006
4-week treatment						
Vehicle	222.3 ± 83.3	44.0 ± 19.8	82.7 ± 38.0	40.8 ± 29.8	23.8 ± 11.1	31.0 ± 23.3
Manumycin A	92.2 ± 13.0	25.2 ± 7.6	39.0 ± 4.9	20.0 ± 4.2	7.0 ± 3.4	1.0 ± 1.1
P value	0.004	0.045	0.016	0.010	0.006	0.003

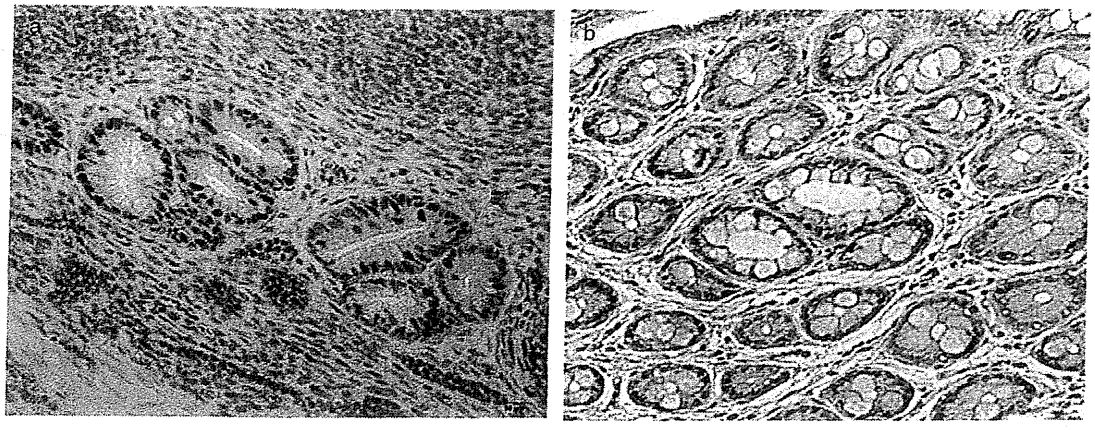


	No. of rats	No. of ACF examined	Positive rate for p-ERK (/100 cell)	
Vehicle	3	30	50.2 ± 19.8	$P < 0.01$
Manumycin A	3	30	13.5 ± 19.2	

Figure 3 Representative immunohistochemical staining for p-ERK in aberrant crypt foci (ACF) from azoxymethane-injected rats. ACF from rats in the vehicle group (a) and in the manumycin A group (b). Strong staining for p-ERK was observed in ACF cells from rats in the vehicle group. However, no staining was observed in ACF cells from rats in the manumycin A group. The positive rate for p-ERK in the manumycin A group was significantly lower than in vehicle group.

the vehicle group. In contrast, no p-ERK expression was observed in the manumycin A group. A total of 30 ACF tissues were evaluated in the manumycin A and vehicle groups (30 per group). The mean p-ERK positive rate in the manumycin A group was 13.5 ± 19.2%, which was significantly lower than in the vehicle group (50.2 ± 19.8%, $P < 0.01$).

In order to examine cell proliferative activity in ACF, immunostaining for Ki-67 was performed using the same specimens. Figure 4 shows representative results of immunostaining for Ki-67 in the manumycin A and vehicle groups. Strong Ki-67 expression in the nucleus of ACF cells was observed in the vehicle group. However, very weak expression of Ki-67 in ACF cells was observed in the manumycin A group. A total of 30 ACF tissues were evaluated in the manumycin A and vehicle groups (30 per group). The positive rate of Ki-67 staining was 2.2 ± 3.4% in the manumycin A group, which was significantly lower than in the vehicle group (14.7 ± 8.2%, $P < 0.01$). These results strongly suggested that manumycin A inhibited signal transduction via the RAS/RAF/MEK/ERK pathway, thereby leading to suppression of cell proliferative activity.



	No. of rats	No. of ACF examined	Positive rate for Ki67 (/100 cell)	
Vehicle	3	30	14.7 ± 8.2	$P<0.01$
Manumycin A	3	30	2.2 ± 3.4	

Figure 4 Representative immunohistochemical staining for Ki-67 in aberrant crypt foci (ACF) from azoxymethane-injected rats. ACF from rats in the vehicle group (a) and the manumycin A group (b). Strong staining for Ki-67 was evident in ACF cells from rats in the vehicle group; however, staining was very weak in ACF cells from rats in the manumycin A group. The positive rate for Ki-67 in the manumycin A group was significantly lower than in the vehicle group.

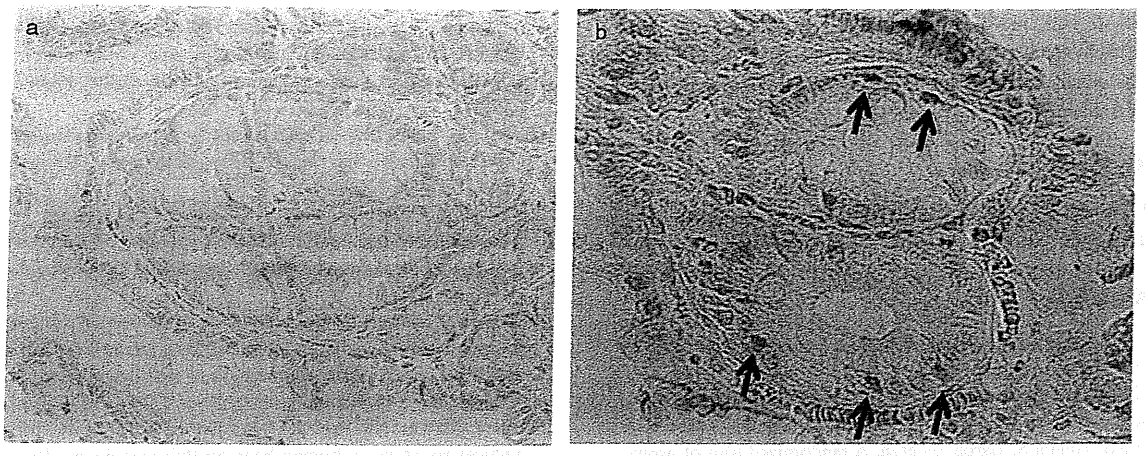
Manumycin A enhances TUNEL positivity in ACF. Because it has been reported that manumycin A induces apoptosis in some cancer cell lines *in vitro*, apoptotic cells in ACF from colorectal tissues obtained, as described earlier, were examined by TUNEL staining. Representative TUNEL staining results are shown in Figure 5. Numerous TUNEL-positive cells with brown nuclei were detected in the manumycin A group, whereas fewer TUNEL-positive cells was observed in the vehicle group. A total of 30 ACF tissues were evaluated in the manumycin A and vehicle groups (30 per group). The mean number of TUNEL-positive cells in the manumycin A group was 8.6 ± 9.7%, which was significantly greater than in the vehicle group (2.9 ± 2.0%). These results indicate that manumycin A not only inhibited cell proliferation signal but also induced apoptosis in ACF tissues.

Discussion

In this study, the RAS inhibitor manumycin A clearly suppressed ACF formation in the AOM-induced colorectal carcinogenesis model, strongly suggesting that the drug has a chemopreventive effect on colorectal cancer. There has been only one previously published investigation of the chemopreventive effect of RAS inhibitors on cancers, that is, a farnesyltranferase inhibitor, FTI276, which lowered the occurrence of lung cancer in a carcinogen-induced mouse model,[27] but there have been no published reports on the preventive effect of RAS inhibitors on other types of cancer, including colorectal cancer. Our data also suggest that RAS inhibitors would potentially be effective as chemopreventive agents not only for colorectal cancer but also for other cancers with RAS mutation including pancreatic cancer, hematological malignancies, and biliary duct cancer.

ACF formation was sufficiently suppressed by not only 8-week administration of manumycin A but also when 4-week administration in the latter half of the study was performed. Because ACF are reported to develop at about 2–4 weeks after AOM administration,[28] our results suggest that manumycin A could potentially eradicate pre-existing ACF. Patients with colorectal adenoma resected endoscopically are considered to be a high-risk group for colorectal cancer and are plausible candidates for cancer chemoprevention. We and other researchers have so far found that a majority of patients with adenoma have numerous ACF.[12,29,30] Moreover, we recently found that short-term administration of sulindac eradicated ACF in polypectomized patients; the occurrence of polyps (adenoma) 1 year later was also significantly suppressed.[31] Therefore, if RAS inhibitors can eradicate pre-existing ACF, it is expected that they could also reduce the occurrence of colorectal adenoma in humans. In this context, it was important to show that manumycin A (a RAS inhibitor) could eradicate pre-existing ACF in this study.

Aside from K-RAS mutation, activation of the PI3kinase/AKT pathway and β-catenin pathway in ACF has been reported in studies using the AOM rodent model.[32–34] Therefore, it is plausible that the ACF that developed in the manumycin A group were negative for K-RAS mutation and associated with activation of



	No. of rats	No. of ACF examined	TUNEL positive rate (/100 cell)	
Vehicle	3	30	2.9 ± 2.0	$P < 0.05$
Manumycin A	3	30	8.6 ± 9.8	

Figure 5 Representative terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining in aberrant crypt foci (ACF) from azoxymethane-injected rats. ACF from rats in the vehicle group (a) and in the manumycin A group (b). Several TUNEL-positive cells with brown nuclei were observed in ACF cells from rats in the vehicle group. However, no TUNEL-positive cells were detected in ACF cells from rats in the manumycin A group. The TUNEL positive rate in the manumycin A group was significantly higher than in the vehicle group.

PI3kinase or β-catenin pathways. Moreover, it has been reported that approximately 30% of ACF show histologically dysplastic features (i.e. high-grade dysplasia) in the AOM rat model.[35] Because dysplastic ACF consist mostly of large-sized ACF, there is possibility that dysplastic ACF were predominantly suppressed by manumycin A, although we did not analyze the precise number of dysplastic ACF in this study. In addition, there was some discrepancy in the number of ACF between the 8-week and 4-week vehicle groups (Table 1), although the difference between the two groups was not statistically significant. It may be explained by systematic (theoretical) experimental errors. Otherwise, it is possible that the vehicle including DMSO might have affected the metabolism of AOM concomitantly administered in the 8-week vehicle group.

As for the underlying mechanism of ACF suppression by manumycin A, the significant decrease of p-ERK and Ki-67 positivity in ACF cells that was observed in manumycin A group suggests that suppression of cell proliferation occurred through inhibition of the RAS/RAF/MEK/ERK pathway. The involvement of this mechanism was supported by the finding that larger sized ACF were more efficiently suppressed. In addition, manumycin A led to a significant increase in the number of TUNEL-positive cells, indicating that it induces apoptosis. This is consistent with our experimental results indicating that pre-existing ACF could be eradicated by administration of manumycin A for 4 weeks during the latter half of the study.

Regarding the mechanism of apoptosis by manumycin A, Pan and associates reported that manumycin A induced the production of reactive oxygen species (ROS), release of cytochrome c, and activation of caspases leading to apoptosis in anaplastic thyroid cancer cells.[36] Similarly, Sears and colleagues reported that manumycin A induced apoptosis via ROS production and subsequent activation of caspases by disruption of MEK and AKT activation in myeloma cell lines.[37] Therefore, it is plausible that in the present study manumycin A induced apoptosis in ACF tissues by stimulating ROS production and subsequent caspase activation.

No clinical study of manumycin A has been reported to date, although it was shown to have insignificant toxicity.[38] RAS inhibitors such as manumycin A may prove to be useful for chemoprevention in future. A clinical trial evaluating a newly developed RAS farnesyltransferase inhibitor, salirasib, for the treatment of pancreatic and lung cancer is currently underway. However, the therapeutic response to date has not been sufficient.[39] Since ACF are minute (< 1 mm in diameter) precancerous lesions and have a very simple genetic mutation (K-RAS mutation but not adenomatous polyposis coli or p53 mutation), it is expected that RAS inhibition will be very effective for eradication. Thus, RAS inhibitors appear most promising as chemopreventive agents rather than cancer treatment agents. However, vemurafenib and dabrafenib, inhibitors of RAF downstream from RAS, are now in clinical use for cancer therapy, and a clinical trial on the MEK inhibitor selumetinib as a cancer therapeutic agent is currently underway.[40]

Because all of these inhibitors eventually suppress p-ERK expression and subsequently cell proliferation, as does manumycin A, these drugs could also be potentially effective for the chemoprevention of colorectal cancer.

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Current status of endoscopic resection strategy for large, early colorectal neoplasia in Japan

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Abstract
Background Conventional endoscopic resection (CER) for early colorectal neoplasia (CRN) is widely accepted as a minimally invasive treatment. Endoscopic submucosal dissection (ESD) was developed in Japan to resect larger lesions, but ESD was not covered by the Japanese national health insurance until April 2012. In addition, treatment strategies vary considerably among medical facilities. To evaluate the current situation in Japan regarding endoscopic treatment of CRNs measuring ≥20 mm, we conducted a prospective multicenter study at 18 medium-volume and high-volume specialized facilities in cooperation with the Japan Society for Cancer of the Colon and Rectum (JSCCR).
Methods The JSCCR conducted a multicenter, observational study of all patients treated by CER and ESD of CRNs measuring ≥20 mm.
Results From October 2007 to December 2010, CERs and ESDs were performed on 1,845 CRNs (CERs 1,029; ESDs 816). Lesions diagnosed as protruded, flat, and depressed totaled 541, 1224, and 48, respectively. En bloc resection rates and mean procedure times for CER/ESD were 56.9 %/94.5 % ($P < 0.01$) and 18 ± 23 min/96 ± 69 min, respectively. The average ESD procedure time was 129 ± 83 min in the ≥40-mm group. As lesion size increased, the CER en bloc resection rate decreased significantly (trend $P < 0.01$), but the ESD en bloc resection rate remained over 93 %. Perforation and delayed bleeding rates of CER/ESD were 0.8 %/1.6 % ($P < 0.05$) and 2 %/2.2 % ($P = 0.3$), respectively.

This study was reported at the United European Gastroenterology Week held at Stockholm, Sweden, October 24, 2011.

This study was conducted on behalf of the Colorectal Endoscopic Resection Standardization Implementation Working Group, Japanese Society for Cancer of the Colon and Rectum, Tokyo, Japan. The working group that participated in this study are listed in "Appendix".

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Conclusions The en bloc resection rate for ESD was significantly higher than for CER, although complication rates were fairly low. Despite a longer procedure time, safety of colorectal ESD has improved in various facilities in Japan. However, ESD for lesions measuring ≥40 mm must be performed by experienced endoscopists due to the longer procedure time.

Keywords Endoscopic mucosal resection · Endoscopic submucosal dissection · Colorectal cancer · Colorectal neoplasia

The number of endoscopic submucosal dissections (ESDs) for colorectal neoplasms has been increasing in Japan, and the effectiveness of colorectal ESD has been reported not only in Japan but also in western countries. However, colorectal ESD still has a higher risk of perforation because the colonic wall is thinner and endoscope stabilization is more difficult than in gastric and esophageal ESD. Consequently, treatment strategies for CRN vary considerably among facilities even in Japan.

Colorectal cancer is a major cause of morbidity and mortality in the world [1]. According to the adenoma–carcinoma sequence theory, early detection and resection of colorectal neoplasm (CRN) is essential for improving cancer mortality [2, 3]. CRNs without risk of lymph node metastasis, including adenomas, are good candidates for endoscopic resection (ER) [4]. Conventional endoscopic resection (CER), including polypectomy and endoscopic mucosal resection (EMR), was developed as a minimally invasive treatment for CRN [5, 6] and is widely accepted. However, CER for lesions exceeding 20 mm in diameter sometimes results in piecemeal resection, decreasing the accuracy of pathological diagnosis and resulting in local recurrences [7–9].

ESD is an established therapeutic technique for the treatment of gastrointestinal neoplasms. Because it is typically completed as en bloc resection, this technique provides a complete specimen for precise histopathological evaluation [10–12]. Following widespread use in treatment of gastric ESDs, the number of medical facilities performing colorectal ESDs has been increasing not only in Japan, but also in western countries [13–21]. However, In the guidelines of the Japanese Society for Cancer of the Colon and Rectum (JSCCR), CRN diagnosed as clinical mucosal cancer or superficial submucosal cancer (invasion depth of <1,000 μm), a size of ≥20 mm was initially recommended for surgical resection [22] because of the greater technical difficulty involved and the risk of perforation and resultant peritonitis [19, 23, 24].

Consequently, treatment strategies (and payment arrangements) for CRN vary considerably among facilities. To evaluate the current situation in Japan regarding endoscopic treatment of CRNs measuring ≥20 mm, we conducted a cross-sectional multicenter study in cooperation with JSCCR. We seek to convey the effectiveness and safety of both CER and ESD treatments to the world.

Materials and methods

From October 2007 to December 2010, patients were prospectively and consecutively enrolled at the 18 institutions affiliated with the Colorectal Endoscopic Resection Standardization Implementation Working Group of JSCCR, and all obtained data were sent to a data center. JSCCR has proposed Japanese guidelines and this working group has a responsibility in ER section of the guideline [19, 20]. The study was conducted with the approval of each institution's ethical review board, and informed written consent was obtained from all patients for each specific colonoscopic treatment. The clinical trial number of this study is UMIN000001642.

We analyzed the following clinicopathological factors: ER method, patient age at the initial ER, sex, tumor size, location, macroscopic type, histological margin, histological grade, depth of submucosal invasion, and lymphatic/venous involvement, determined based on the Japanese classification of cancer of the colon and rectum (JCCCR) [22].

All procedures were performed by experienced colonoscopists, or under their supervision, with a standard videoendoscopic system (EVIS LUCERA system, Olympus Optical, Tokyo, Japan; or Advancia HD/Advancia, Fujifilm, Tokyo, Japan).

Inclusion criteria

ER is indicated to treat intramucosal CRNs and lesions with submucosal invasion limited to less than 1,000 μm, because the risk of lymph node metastasis is very low [4, 25]. Before treatment, only depth of invasion could be estimated endoscopically in combination with conventional endoscopic findings and, if possible, pit pattern analysis with magnifying chromoendoscopy (CF-H260AZI, CF-Q260AZI, or PCF-Q240ZI, Olympus, Tokyo, Japan; and EC590Z series, Fujifilm, Tokyo, Japan) [26–32]. We have indicated the use or nonuse of magnification.

The Colorectal ESD Standardization Implementation Working Group has attempted to standardize colorectal ESD, and guidelines have been proposed by this group

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[19, 20]. Based on extensive clinicopathological analyses, the indications for colorectal ESD in this study are the same as those recommended in the guidelines: a tumor for which the use of a snare EMR for en bloc resection is difficult, such as a laterally spreading tumor of the nongranular type (LST-NG) [7, 20, 33, 34], especially the pseudo-depressed type, a tumor with a type V_I pit pattern, a shallow infiltrating submucosal carcinoma, a large depressed tumor, and large elevated, probably malignant lesions (tall nodule-aggregating lesions such as a granular-type LST; LST-G), because these lesions have a high submucosal invasion rate and are difficult to treat even by piecemeal EMR [19, 33, 35]. Other lesions, such as intramucosal tumors accompanied by submucosal fibrosis, which are induced by a biopsy or peristalsis of the lesion, sporadic localized tumors in chronic inflammation, including ulcerative colitis, and local residual early carcinomas after EMR, also are indications for colorectal ESD [19].

Exclusion criteria for ER

Exclusion criteria included findings of submucosal cancer such as V_N pit pattern, an invasive pattern as determined by magnification chromoendoscopy [27, 29, 36], and presence of other invasive cancers and circumferential tumors that require surgical treatment because of the increased technical difficulty involved and the anticipated risk of stenosis.

Clinicopathological characteristics

The location of tumors was based on the Japanese classification of cancer of the colon and rectum [22, 37] and included the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum.

For macroscopic typing, we divided the lesions into five macroscopic groups according to the Paris classification and LST features as follows: (1) protruded type, which is 0-Is(p); (2) flat type, which is 0-IIa and 0-IIc with LST features; (3) mixed type, 0-Is(p) + IIa, most of which have LST character; (4) depressed type, 0-IIc or IIa + IIc in Paris classification without LST features; and (5) recurrent cases.

CER procedures

In this study, CER was defined as snare technique, EMR, or snare polypectomy; endoscopists, including gastroenterologists and digestive surgeons, chose treatment methods according to the size and endoscopic features of the CRN. In EMR, after successful fluid injection of normal saline and/or glycerol [38] and/or 0.4 % hyaluronic acid solution into the submucosal layer, the endoscopist performed resection using the snare [6]. After resection, additional snare resection or coagulation using hot biopsy forceps also was performed if there was a suspicion of small residual tumors in the resection plane.

ESD procedures

Procedures were primarily performed using one or two ESD knives, including a bipolar needle knife (Xeon Medical Co, Tokyo, Japan) [9, 15, 39], flex knife [40], hook knife (Olympus Co, Tokyo, Japan) [41], flush knife (Fujinon Co, Tokyo, Japan) [42], and insulation-tipped knife (Olympus) [10, 13]. Hemostatic forceps (Coagrasper; Olympus) and Hemostat Y (PENTAX Co., Tokyo, Japan) were used for hemostasis. Lesion margins were delineated before ESD using 0.4 % indigo-carmine spray dye. Following injection of Glycerol and/or sodium hyaluronate into the submucosal layer, a circumferential incision was made using the ESD knife [14]. Both partial circumferential incision and subsequent submucosal dissection were performed alternately using ESD knives.

Definition of ESD and CER

Some lesions were treated by a combined CER/ESD technique, using a special ESD knife and resected by snaring. We defined those cases of resection by snaring with only circumferential incision [43] as CER and cases in which the physician performed any submucosal dissection after marginal resection as ESD.

Definition of complication

Perforation during an ESD procedure was defined as immediate, and delayed perforation was defined as any perforation occurring after completion of the procedure. Immediate perforation was defined as a full-thickness defect in the colonic wall. Closure with endoscopic clips was performed or surgical treatment was pursued. Postoperative bleeding was defined as bleeding that required repeat colonoscopy for hemostasis therapy, blood transfusion, or decreased level of hemoglobin >2 g/dl.

Histological assessment

All specimens were fixed in 10 % formalin, cut into 2-mm sections, and examined microscopically for histological type, depth of invasion, and lateral and vertical resection margins. Resections were considered tumor-free when the lateral and vertical margins of a specimen were both negative for tumor cells, independent of histological features. The submucosal depth was defined as the distance determined by microscopic observation of specimens using an optical micrometer [4]. A curative resection was achieved when both the lateral and vertical margins of the specimen were free of cancer, with none of the following features: submucosal invasion deeper than 1,000 μm, lymphatic invasion, vascular involvement, or poorly differentiated

components [4]. An adenoma with an unknown lateral margin also was considered to be resected curatively when the neoplasm met all other criteria. Lesions resected in a piecemeal fashion were reconstructed faithfully on the basis of the mirror endoscopic images obtained before treatment and fixed in formalin. Histological diagnoses were based on the Japanese classification of cancer of the colon and rectum [37] and the Vienna classification [44]. The former is a standard pathological classification in Japan, and these results were converted to the latter form for standardization with global classifications.

Statistical analysis

Statistical analysis was performed using SPSS version 20 (SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation, medians, ranges, and percentages. For analysis of clinicopathological characteristics, we used Student's t test and χ^2 and Fisher's exact tests, as appropriate. All tests were two-tailed, and $P < 0.05$ was considered significant.

Results

Patient and lesion characteristics

A total of 1,845 CRNs that were ≥20 mm in size were examined in this study. CER was used in 1,029 cases and ESD in 816. Mean lesion sizes in CER and ESD cases were 26.4 ± 8.6 (range 20–120) mm and 39.4 ± 18.2 (range 20–174) mm, respectively. Patient characteristics and distributions of lesions are detailed in Tables 1 and 2. Tumor distribution between the two groups was different ($P < 0.01$). The frequency of cecum and sigmoid colon lesions were higher in the CER group than in the ESD group. On the other hand, lesions of the rectum were less frequent in the CER group. Submucosal cancer, including both superficial submucosal cancer and deep submucosal invasion cancer, was more common in the ESD group. Thus, the distribution of tumor characteristics differed between CER and ESD. The frequency of use of magnification colonoscopy also differed in the two groups (71.9 % in CER vs. 85.7 % in ESD, $P < 0.01$).

Differences in endoscopic treatment choice according to tumor size and macroscopic type

We divided the cases into three groups according to lesion size: 20–29, 30–39, and ≥40 mm. Associations between tumor size, macroscopic type, and treatment choice are detailed in Table 3. In the 20–29-mm group, 77 % (729/948) of the lesions were treated by CER. In contrast, as the lesion size increased, lesions were more likely to be treated by ESD. Macroscopic type also influenced treatment choice. In the 20–29-mm group, 93.8 % of the protruded lesions were treated by CER, whereas 37 % of the flat lesions were treated by ESD. In the 30–39-mm group, approximately 70 % of mixed and flat lesions were treated by ESD. In the ≥40-mm group, approximately 80 % of mixed and flat lesions were treated by ESD. All five cases of recurrence were treated by ESD, regardless of lesion size.

Table 1 Patients and tumor location

	CER	ESD	Total	*P* value
No. of lesions	1,029	816	1,845	
Age, mean ± SD (range)	65.2 ± 11.7 (20–89)	66.6 ± 9.9 (23–91)	65.8 ± 10.9	<0.01
Sex, male/female (ratio)	637/392 (1.6)	468/348 (1.3)	1105/740 (1.5)	<0.05
Use of magnification (%)	740 (71.9)	700 (85.7)	1440 (78)	<0.01
Distribution				
Cecum (%)	137 (13.3)	71 (8.7)	208 (11.3)	
Ascending colon (%)	231 (22.4)	152 (18.6)	383 (20.8)	
Transverse colon (%)	161 (15.6)	144 (17.6)	305 (16.5)	
Descending colon (%)	38 (3.7)	32 (3.9)	70 (3.8)	
Sigmoid colon (%)	300 (29.2)	121 (14.8)	421 (22.8)	
Rectum (%)	162 (15.7)	296 (36.3)	458 (24.8)	<0.001
Total (%)	1,029 (100)	816 (100)	1,845 (100)	

Table 2 Pathological results, by procedure type and lesion size

Range of lesion sizes (mm)	20–29 CER/ ESD	30–39 CER/ ESD	≥40 CER/ ESD	Total CER/ ESD	Total
Pathological results					
Adenoma	380/72	86/95	36/95	502/262	764
(%)	(84/16)	(48/52)	(27/73)	(66/34)	
Intramucosal cancer	283/95	85/109	65/194	433/398	831
(%)	(75/25)	(44/56)	(25/75)	(52/48)	
Submucosal cancer	48/52	17/51	5/47	70/150	220
(%)	(48/52)	(25/75)	(10/90)	(32/68)	
<1,000 μm	18/34	8/31	3/23	29/88	117
(%)	(35/65)	(21/79)	(12/88)	(25/75)	
≥1,000 μm	30/18	9/20	2/24	41/62	103
(%)	(63/38)	(31/69)	(8/92)	(40/60)	
Unknown	0/0	0/1	0/2	0/3	3
(%)	(0/0)	(0/100)	(0/100)	(0/100)	
Others	18/0	6/2	0/1	24/3	27
(%)	(100/0)	(75/25)	(0/100)	(89/11)	
Total	729/219	194/258	106/339	1029/816	

Treatment results: comparison of CER and ESD

The treatment results for CER and ESD are detailed in Table 4. Operation time for ESD was much longer than for CER, although the en bloc resection rate was significantly higher in the ESD group. We found that procedure time and tumor size were associated, especially in ESD cases. Compared with the CER cases, as lesion size increased, the ESD procedure time increased.

Even in the 20–29-mm group, the en bloc resection rate for CER was only 66.5 %, which is significantly lower than that of the ESD group. As lesion size increased, the en bloc resection rate for CER decreased; the en bloc resection rate in the ≥40-mm group was only 12.3 %. In contrast, the en bloc resection rate for ESD was maintained at >93 %, even in the ≥40-mm group.

Complication rate

The number of delayed bleeding cases was 18 (1.7 %) in the CER group and 18 (2.2 %) in ESD ($P = 0.3$). The number of perforation cases in these groups was 8 (0.8 %) and 16 (2 %; $P < 0.05$), respectively. The ESD perforation rate was higher than the CER rate, but most ESD and CER perforation cases were successfully treated endoscopically; only three cases (1 CER, 2 ESD) required emergency surgery.

Table 3 Macroscopic type of lesion, by procedure type and lesion size

Range of lesion sizes (mm)	20–29 CER/ESD	30–39 CER/ESD	≥40 CER/ESD	Subtotal CER/ESD	Total
Lesion number	729/219	194/258	106/339	1,029/816	1,845
(%)	(23)	(57)	(76)	(44)	
Macroscopic type					
Protruded	363/24	87/27	25/17	475/68	543
(%)	(6)	(24)	(40)	(13)	
Mixed	88/28	39/86	56/220	183/334	517
(%)	(24)	(69)	(80)	(65)	
Flat	275/164	68/144	25/101	368/409	777
(%)	(37)	(68)	(80)	(53)	
Depressed	3/0	–/–	–/–	3/0	3
(%)	(0)			(0)	
Recurrence	0/3	0/1	0/1	0/5	5
(%)	(100)	(100)	(100)	(100)	

Table 4 Treatment results by procedure type and lesion size

Range of lesion sizes (mm)	CER			ESD			CER	ESD	Total
	20–29	30–39	≥40	20–29	30–39	≥40	Sub total		
Lesion number	729	194	106	219	258	339	1029	816	1,845
Procedure time (min, mean ± SD)	13 ± 13	43 ± 23	42 ± 46	66 ± 45	79 ± 42	129 ± 83	18 ± 23	96 ± 69	53 ± 63
Complication									
Delayed bleeding	12	4	2	3	7	8	18	18	36
(%)	(1.6)	(2.1)	(1.9)	(1.4)	(2.7)	(2.4)	(1.7)	(2.2)	(2)
Perforation	5	3	0	4	7	5	8	16	24
(%)	(0.7)	(1.5)	(0)	(1.8)	(2.7)	(1.5)	(0.8)	(2.0)	(1.3)
Emergency surgical operation	–	1	–	–	–	2	1	2	3
(%)		(0.5)				(0.6)	(0.1)	(0.2)	(0.2)
En bloc resection rate	485	88	13	206	248	317	586	771	1,357
(%)	(66.5)	(45.4)	(12.3)	(94.1)	(96.1)	(93.5)	(56.9)	(94.5)	(73.6)
Non-curative resection	33	9	2	23	24	31	44	77	122
(%)	(4.5)	(4.6)	(1.9)	(10.5)	(9.3)	(9.1)	(4.3)	(9.4)	(6.6)
Additional surgery	29	9	3	17	22	23	41	62	103
(%)	(4.0)	(4.6)	(2.8)	(7.8)	(8.5)	(6.8)	(4.0)	(7.6)	(5.6)

Pathological results and additional surgery

Histopathological assessment led to the diagnosis of 44 (4.3 %) CER cases and 77 (9.4 %) ESD cases as noncurative resections. Furthermore, 41 CER patients (4 %) and 62 ESD patients (7.6 %) underwent additional surgery.

Discussion

Key findings

In this prospective, multicenter study in Japan, we surveyed the current status of endoscopic treatment for relatively large CRNs (≥20 mm). As size increased, Japanese endoscopists were more likely to select ESD, especially for the treatment of flat- and mixed-type CRNs. As a result, the en bloc resection rate for ESD was significantly higher than for CER, although complication rates were very low in both groups. Despite longer procedure time, ESD is becoming safe and is considered a standard procedure in Japan for the treatment of large, superficial CRNs.

Before this study, Saito et al. reported the results of an initial, prospective, multicenter cohort study of ESD [18]. They analyzed the results of all colorectal ESD cases from the time of introduction of the procedure, performed at ten specialized facilities ($n = 1{,}111$). By contrast, the present study was planned after approval of ESD with advanced medical care systems, with a strict treatment indication; therefore, both highly advanced medical facilities and general facilities participated, enrolling the cases in a limited research registration period. In our study, the overall perforation rate was only 1.3 % ($n = 24$), and the rate of emergency surgery was extremely low (0.3 %, $n = 3$) compared with the previous study [18], suggesting improved safety of colorectal ESD in various facilities.

Japanese guidelines for ER for colorectal cancer and ESD indication

According to the guidelines of the JSCCR, CRNs diagnosed as clinical mucosal cancer or superficial submucosal cancer (<1,000 μm) are indicated for ER. However, 20 mm is the largest size of a tumor that can be easily resected en bloc by polypectomy or snare EMR. If the preoperative diagnosis is adenoma or carcinoma in adenoma, piecemeal resection can be performed. It should be noted, however, that piecemeal resection is associated with a high incomplete resection rate and a high local recurrence rate. Therefore, such lesions were a relative indication for surgical resection [22]. After introduction of the ESD technique for CRN treatment, it became possible to perform en bloc resections for lesions measuring > 20 mm. Our study shows that Japanese endoscopists selected ESD rather than CER, as tumor size increased, especially for mixed- and flat-type CRNs. Endoscopists make this choice, because they know the pathological character of large CRNs, which incur the risk of noncurative resection contrary to pre-ESD expectations and because they know the indication of colorectal ESD.

Treatment selection and outcome as related to tumor size

In this study, we performed endoscopic treatment according to the guidelines of JSCCR and indication of ESD. As size increased, selection of ESD became more common, perhaps because Japanese endoscopists understand the difficulty of performing en bloc resection for larger CRNs by CER.

However, ESD has a big limitation. As tumor size increased, procedure time increased, compared with CER. Our data showed that the average procedure time for colorectal ESD for lesions measuring ≥40 mm was more than 2 h. It should be noted that such lesions were treated by surgery before ESD became widespread; therefore, it may be more informative to compare ESD procedure time with that of surgical cases instead of EMR cases. ESD for CRNs measuring ≥40 mm is thought to be a difficult and time-consuming treatment, so we recommend that ESD for lesions <40 mm be considered a general procedure but that lesions measuring ≥40 mm should be treated in medical facilities with more experienced staff.

Treatment selection as related to tumor macroscopic type

In the 20–29-mm group, protruded-type CRNs were likely to be treated by CER. However, the proportion of CER for mixed and flat lesions was less than that for protruded type. As the lesion size increased, mixed and flat lesions were more likely to be treated by ESD. As the proportion of the flat component in the CRN groups increased, the proportion of ESD increased.

En bloc resection rate and complication rate

We found that the en bloc resection rate for ESD was significantly higher than for CER, although complication rates in both groups were quite low in these representative Japanese facilities. The ESD technique enabled complete resection even for the large-sized tumors. This may indicate that recent improvements in endoscopic devices and instruments have reduced complications (such as perforation) in ESD. Most perforation cases were managed

endoscopically; only two ESD cases required emergency surgical treatment. Due to these improvements, the ESD technique is now widely accepted for the management of large CRNs in Japan.

Considering the learning curve for colorectal ESD, in a previous study, we retrospectively reviewed clinical outcomes of colorectal ESD performed by trainees. Under the guidance of experienced specialists, trainee endoscopists are able to perform colorectal ESD without serious complications after preparatory training and experience with ≥30 cases [45]. Saito et al. [18] reported that the complication rate was 17.6 % at medical facilities in which the number of ESDs performed was less than 50. Univariate and multivariate analysis revealed that large tumor size (>50 mm) and less experience performing ESDs (<50 cases) were independent risk factors for complications [15]. Tanaka et al. [19] reported that the perforation rate in colorectal ESD decreased annually with experience.

Multicenter studies of ER have been conducted outside Japan. Moss et al. [46] reported outcomes of ER for lesions more than 20 mm, in an important Australian prospective, multicenter, observational study. They concluded that EMR is a safe and effective therapy for large sessile polyps. Some differences between their study and ours include the following: (1) their study reported only EMR results; (2) the percentage of submucosal cancers in their study was relatively low compared with our study (33 cases, 6.9 % and 220 cases, 11.9 %, respectively). Based on these differences, we assume that the Australian group referred some cases directly for surgical resection, whereas we may perform ESD as the first treatment. Long-term follow up evaluation was not extensive in both studies.

Saito et al. [9] reported the results of long-term follow-up after EMR and ESD and described one recurrence case as invasive cancer after 2.5 years; the case was histologically diagnosed in the first EPMR as a curative resection. In an evaluation of one leading Japanese hospital, Kobayashi et al. [47] reported that after introduction of colonic ESD, use of ER was widespread and reduced the incidence of repeat surgery for large-size intramucosal cancer.

Moss et al. also concluded that lesions having a high possibility of submucosal deep invasion, such as LST-NG or lesions with advanced pit pattern, should be treated en bloc to achieve accurate pathological assessment. In light of these findings, our study shows that ESD is becoming a standard procedure for en bloc resection in Japan.

Limitations

The major strength of our study is the large sample size; however, our study has some limitations. First, this was a prospectively enrolled, multicenter cohort study, not randomized; thus, eligibility criteria for performing colorectal ESDs were sometimes unclear at some institutions. Until the end of 2011, ESD was performed in more than 180 medical facilities in Japan as a generalized technique; therefore, randomization between CER and ESD was considered too difficult. Second, the recurrence rate of CER and ESD 1 year after initial endoscopic treatment is an important goal of this working group study; therefore, we will analyze those data and report them elsewhere. For the prescription of colorectal ESD by Japanese national health insurance, an additional nationwide survey to assess the clinical outcomes of colorectal ESD is recommended.

Conclusions

The en bloc resection rate for ESD was significantly higher than that for CER regardless of the tumor size; otherwise, submucosal cancer was more common in the ESD group. In addition, there was no significant difference in complications between the two groups. Our study proves that ESD is a feasible treatment for patients with mucosal CRN >20 mm, although long-term outcomes should be evaluated in the future. Such findings were presented at the annual meeting of United European Gastroenterology Week, 2012. For en bloc resection of lesions measuring ≥40 mm, ESD is an essential technique, but the procedure must be performed by experienced endoscopists in well-equipped medical facilities, because the procedure time is long.

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Appendix

Facilities that participated the study

The patients were enrolled at the 18 institutions affiliated with the Colorectal Endoscopic Resection Standardization Implementation Working Group of JSCCR as follows: (1) Endoscopy Division, National Cancer Center Hospital, Tokyo, Japan (Takeshi Nakajima, Yutaka Saito, Takahisa Matsuda); (2) Department of Endoscopy, Hiroshima University Hospital, Hiroshima, Japan (Shinji Tanaka); (3)

Department of Gastrointestinal Oncology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan (Hiroyasu Iishi); (4) Digestive Disease Center, Showa University Northern Yokohama Hospital, Kanagawa, Japan (Shin-ei Kudo); (5) Department of Gastroenterology & Gastrointestinal Oncology, National Cancer Center Hospital East, Chiba, Japan (Hiroaki Ikematsu); (6) Department of Endoscopy, Cancer Institute Ariake Hospital, Tokyo, Japan (Masahiro Igarashi); (7) Digestive Disease Center, Asahikawa City Hospital, Hokkaido, Japan (Yuusuke Saitoh); (8) Institute of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan (Yuji Inoue); (9) Department of Gastroenterology, Kitasato University East Hospital, Kanagawa, Japan (Kiyonori Kobayashi); (10) Department of Gastroenterology, Fukuoka University Chikushi Hospital, Fukuoka, Japan (Takashi Hisasbe); (11) Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Fukuoka, Japan (Osamu Tsuruta); (12) Gastrointestinal Center, Sano Hospital, Hyogo, Japan (Yasushi Sano); (13) Department of Gastroenterology, Akita Red Cross Hospital, Akita, Japan (Hiro-o Yamano); (14) Department of Gastroenterology, JR West Osaka Railway Hospital, Osaka, Japan (Seiji Shimizu); (15) Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan (Naohisa Yahagi); (16) Department of Surgery, Teikyo University Hospital, Tokyo, Japan (Toshiaki Watanabe); (17) Department of Gastroenterology, Chofu Surgical Clinic, Tokyo, Japan (Hisashi Nakamura); (18) Gastroenterology, Takahiro Fujii Clinic, Tokyo, Japan (Takahiro Fujii).

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