In vitro analysis of exfoliated tumor cells in intraluminal lavage samples after colorectal endoscopic submucosal dissection

Takayuki Nakamoto[1,2] · Fumikazu Koyama[1,2] · Hiroyuki Kuge[1] · Shinsaku Obara[1] · Naoya Ikeda[1] · Yosuke Iwasa[1] · Takeshi Takei[1] · Tomomi Sadamitsu[1] · Suzuka Harada[1] · Kosuke Fujimoto[1] · Takashi Inoue[1] · Kinta Hatakeyama[3] · Chiho Ohbayashi[3] · Masayuki Sho[1]

Departments of [1]Surgery, [2]Division of Endoscopy and [3]Department of Diagnostic Pathology, Nara Medical University, Nara, Japan

Correspondence to: Fumikazu Koyama

Department of Surgery, Nara Medical University

840 Shijo-cho, Kashihara, Nara, 634-8522, Japan.

E-mail: fkoyama@naramed-u.ac.jp

Tel: +81 744223051, Fax: +81 744246866,

Abstract

Purpose Colorectal endoscopic submucosal dissection (ESD) produces exfoliated tumor cells that occasionally cause local recurrence. However, the biological characteristics of these tumor cells have not been clarified. The aim of this study was to clarify the genetic background and viability of exfoliated tumor cells in colorectal ESDs, as well as possible method for their elimination.

Methods Post-ESD intraluminal lavage samples from 19 patients who underwent colorectal ESDs were collected. In four patients with adenocarcinoma, gene mutations in the primary tumors and exfoliated cells in lavage samples were analyzed using a next-generation sequencer (NGS). In 15 patients with adenoma or adenocarcinoma, the viability of exfoliated cells and the cell-killing effect of povidone-iodine on exfoliated cells were evaluated.

Results The analysis using a NGS demonstrated that tumors targeted for ESD had already acquired mutations in many genes involved in cell proliferation, angiogenesis, and invasions. Furthermore, gene mutations between the exfoliated tumor cells and tumors resected by ESDs showed a 92% to 100% concordance. The median viable cell counts and the median viability of exfoliated cells in intraluminal lavage samples after ESDs were 4.9×10^5 cells/mL and 24%, respectively. The viability of the exfoliated cells did not decrease even 12 hours after ESD. However, contact with 2.0% povidone-iodine solution reduced both viable cell counts and viability, significantly.

Conclusion A large number of tumor cells exfoliated during colorectal ESDs had acquired survival-favorable gene mutations and could survive for some time. Therefore, a lavage using a solution of 2.0% povidone-iodine may be effective against such cells.

Trial registration number: The prospective study registered 1317, and the retrospective study registered 2729.

Trial registration date of registration: The prospective study approved on June 20, 2016, and the retrospective study approved on October 6, 2020.

Keywords: endoscopic submucosal dissection · colorectal neoplasm · mutation · cell survival · therapeutic irrigation

Declarations

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Authors Contributions: All authors contributed to the study conception and design. Material preparation and data collection were performed by Takayuki Nakamoto, Fumikazu Koyama, Hiroyuki Kuge, Shinsaku Obara, Yosuke Iwasa, Takeshi Takei, Tomomi Sadamitsu, Suzuka Harada, Kosuke Fujimoto, Takashi Inoue. Histological examinations were performed by Kinta Hatakeyama and Chiho Ohbayashi. Data analysis and interpretation were performed by Takayuki Nakamoto, Fumikazu Koyama, Hiroyuki Kuge, Shinsaku Obara, Naoya Ikeda, Kinta Hatakeyama, Chiho Ohbayashi, and Masayuki Sho. The first draft of the manuscript was written by Takayuki Nakamoto, Fumikazu Koyama, and Naoya Ikeda, and critical revision of the manuscript was performed by Fumikazu Koyama, Takayuki Nakamoto and Masayuki Sho. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Introduction

The ideal endoscopic treatment for a colorectal tumor is an en bloc resection with negative horizontal and vertical margins [1]. However, there is a limit to the size of the tumors that can be resected at any given time during an endoscopic mucosal resection (EMR), and large tumors require piecemeal resections. Furthermore, a piecemeal resection is the most important risk factor for local recurrences not only in malignant tumors but also in benign tumors [2]. Nonetheless, an endoscopic submucosal dissection (ESD) allows for an en bloc endoscopic resection of the tumor, regardless of size [3, 4].

Colorectal ESDs have been reported to be safe and feasible, although there have been problems such as post-ESD bleeding and perforations (0 – 12% and 1.4 – 10.4%, respectively) [5]. In Japan, ESDs are the standard treatment for lesions for which en bloc resections may be difficult or for very large lesions that carry a high risk of high-grade dysplasias or invasive cancers [3]. ESDs have also been included in the recent European and US guidelines [6, 7]. ESDs have succeeded in reducing the local recurrence rate from approximately 20% with conventional piecemeal resections to less than 2% for large lesions [8-11]. Currently, colorectal ESD is widely recognized as a less invasive treatment than surgical resection, when the possibility of lymph node metastasis is small.

However, local recurrence occurs occasionally, even with en bloc resections by ESD, that is, the oncological problem of a local recurrence still remains [9]. Even when an ESD with an en bloc resection has been achieved, there are at least two possible mechanisms for a local recurrence. One is the possibility of a microscopic residual tumor in and around the ulcer bed after an ESD. The other is the implantation of the exfoliated tumor cells in the large ulcer bed that lacks the normal mucosal barrier [12], due to the ESD.

The authors of this study previously reported a case of local recurrence that may have been due to the implantation of tumor cells after curative resection with a rectal ESD and other authors have also reported similar cases [13-15]. In the same study, tumor cells were discovered in both malignant and benign tumors from the intraluminal lavage samples after colorectal ESDs [16]. Furthermore, it was found that the exfoliated tumor cells could be eliminated with an intraluminal lavage after an ESD, however, the effective elimination of tumor cells required 1000 mL or more of lavage fluid [17].

In order for the malignant and benign tumor cells that are exfoliated during ESD to grow into recurrent tumors, they must have the ability to survive, engraft, and proliferate after exfoliation. Previous studies have not clarified the gene mutations acquired by the tumors targeted for ESDs, whether the gene mutations differed between the primary tumor and the exfoliated cells, whether the exfoliated cells could survive, and whether there is a more effective method for eliminating tumor cells other than with massive intraluminal lavages.

Therefore, the aims of this study were to clarify the following three points:

1. The genetic background of the primary tumors subjected to ESDs and the exfoliated cells.
2. The quantity of the exfoliated cells alive after ESDs and how long they can survive.
3. The *in vitro* cytocidal effect of povidone-iodine solutions against these exfoliated cells.

Methods

Study type and ethical approval

The present study included a retrospective analysis using a next-generation sequencer (NGS) as well as prospective research to evaluate the viability of exfoliated cells after ESD and the cell-killing effect of povidone-iodine on such cells. The protocols for both studies were approved by the Institutional Review Board of Nara Medical University (Ethical Approval Numbers: 1317 and 2729, respectively) and they comply with the provisions of the Declaration of Helsinki. The prospective study was registered with the University Hospital Medical Information Network (UMIN) (identifier: UMIN000026206).

Patients who underwent colorectal ESDs at Nara Medical University Hospital between February 2017 and December 2018 were enrolled in this study. Patients who were unable to undergo en bloc resections or had non-epithelial tumors were excluded from these studies. Post-ESD intraluminal lavage samples and clinicopathological data were collected from 19 patients. The clinicopathological data included age, sex, tumor locations, macroscopic classifications of the tumors, maximum tumor diameters, the time required for the ESDs, histological diagnoses, complications, and the quality of the resections (en bloc resections, resections with negative resection margins, and curative resections). In four patients, gene mutations in the primary tumors and the exfoliated cells from the lavage samples were analyzed using a next-generation sequencer (NGS), retrospectively. In 15 patients, the number and viability of exfoliated cells and the cell-killing effect of povidone-iodine on exfoliated cells were evaluated, prospectively. Written informed consent was obtained from all the patients for the use of the data, the obtained sample, and the publication of the present study.

Pretreatment

The ESD pretreatment diet was a low-residue diet, commonly used when barium enemas are administered. In addition, the patient received a bottle (10 mL) of sodium picosulfate solution, orally, before bedtime on the previous day. The patients were also given a 2 L polyethylene glycol solution (sodium chloride, potassium chloride, sodium carbonate, sodium bicarbonate, anhydrous sodium sulfate, and sodium sulfate), orally. If necessary, additional polyethylene glycol solution was allowed, up to a maximum volume of 4 L.

ESD procedure and intraluminal lavage sampling

The ESD was performed as previously described [15, 16]. An endoscope (CF-Q260AI, CF-H260AZI, or GIF-Q260J; Olympus, Tokyo, Japan, or EW-590ZW; Fujifilm Co., Tokyo, Japan) with a hood, a 1.5-mm ball-tipped flush knife (Flush Knife-BT; Fujifilm Co., Tokyo, Japan) and an electrosurgical unit (VIO200D; Erbe Elektromedizin, Tübingen, Germany)(setting for the mucosal incision: end cut I, effect 2, interval 3, and duration 3; setting for the submucosal dissection: forced coagulation mode, effect 2, and 40 W), were used. A 1% hyaluronic acid solution (MucoUp; Johnson & Johnson, Tokyo, Japan) mixed with a small amount of 0.1% adrenaline and indigo carmine which was diluted 1.5 times with saline, was administered. After the resection of the tumor, 30 mL saline was delivered through the endoscopic forceps channel, focusing on the dissection plane. The lavage fluid was aspirated using endoscopic suction and collected into a trap normally used for stool cultures.

An en bloc resection was defined as a one-piece resection of the entire lesion that was observed endoscopically. Patients with curative resections were defined as those satisfying all of the following criteria based on the Japanese Society for Cancer of the Colon and Rectum Guidelines 2019 (for physicians) for the treatment of colorectal Cancer

[18]: negative horizontal and vertical margins, depth of submucosal invasion of less than 1,000 μm, negative vascular invasions, papillary or tubular carcinomas, and grade 1 budding at the sites of deepest invasion.

Analysis of gene mutations in primary tumors and exfoliated tumor cells

DNA samples were extracted from the resected specimens and lavage samples in four patients with positive tumor cytology. Each extracted sample was analyzed using a NGS (Ion Proton system; Thermo Fisher Scientific, Inc., Waltham, MA) with AmpliSeq for Illumina Cancer HotSpot Panel version 2, to identify somatic mutations across the hotspot regions of 50 genes (Table 1) with known associations to cancer including colorectal cancer, as identified in the Catalogue of Somatic Mutations in Cancer (COSMIC) database [19].

Evaluation of exfoliated cell viability

The lavage samples were assessed at four different time points: 0, 3, 6, and 12 h after the ESD. The lavage samples were stored at room temperature. The viability of the exfoliated cells in the lavage samples was evaluated using trypan blue staining [20]. The procedures for the evaluation of the viability were as follows: 10 μl of lavage sample was mixed with 10 μl of trypan blue and injected by pipette into a disposable cell counting chamber slide (Invitrogen Countess Cell Counting Chamber Slides; Invitrogen, Waltham, MA). The slide was inserted into an automated cell counter (Countess; Invitrogen, Waltham, MA, USA), and the viability was examined. We analyzed the association between cell counts or viability and tumor characteristics including macroscopic appearances, tumor maximum diameters, the time required for the ESDs, histological types (adenomas or carcinomas), cytology statuses (positive or negative), and tumor locations (right side:

cecum to the transverse colon or left side: descending colon to rectum). The macroscopic appearance was classified based on the Paris endoscopic classification of superficial neoplastic lesions in the digestive tract [21].

In vitro treatment with povidone-iodine solutions against exfoliated cells

The cell viability in the lavage samples at the end of the ESD was evaluated in four different concentrations of povidone-iodine (0%, 0.5%, 1.0%, and 2.0%) prepared using 10% povidone-iodine (Kenei Pharmaceutical Co., Osaka, Japan). For example, the procedures for the evaluation of the viability in 0.5% povidone-iodine solution were as follows: 9.5 μL of the sample was mixed with 9.5 μL of trypan blue and 1 μL of 10% povidone iodine, and the viability was evaluated using the same method described previously. Similarly, the viability was evaluated using 1.0% and 2.0% povidone-iodine solutions.

Statistical analysis

Statistical calculations were performed using SPSS version 19.0 (SPSS Inc., Chicago Ill., USA). Data were presented as medians with ranges. Statistical analyses were performed using the Mann-Whitney U test, χ^2 test, or the Fisher's exact test. Statistical significance was accepted for P-values <0.05. To evaluate the association, a Pearson's correlation coefficient was calculated, and statistical correlation was accepted for r values of >0.4.

Results

Gene mutation profile analysis using an NGS

The genetic background of the tumors subjected to ESDs and the exfoliated cells, and the gene mutations in four patients with rectal adenocarcinomas were analyzed using the NGS. Cases 1 and 4 had T1 cancer with venous and lymphatic invasions, while Cases 2 and 3 had Tis cancer (according to the 8th edition of AJCC/UICC TNM classification). AmpliSeq for Illumina Cancer HotSpot Panel version 2 was used to examine 50 genes. Details of the gene mutation profiles are shown in Table 2. In eight samples of four cases, a total of 31 mutations were detected in 23 genes. Mutations were found in 10 genes in Case 1, 16 genes in Case 2, and 14 genes in Cases 3 and 4. All four cases commonly had actionable gene mutations in *APC*, *PDGFRA*, *KDR*, and *FLT3*. In addition, patients with T1 cancer had *KIT* and *SMAD4* mutations in Case 1 and *RAS* and *TP53* mutations in Case 4, as actionable mutations. Patients with Tis cancer had *FBXW7*, *NOTCH1*, *ATM*, *TP53* mutations in Case 2 and *MLH1*, *RET*, *PTEN*, *TP53* mutations in Case 3 as actionable mutations. The evaluation of the gene mutations between the exfoliated tumor cells and the resected tumors showed a 98% concordance in Case 1 and 92% concordance in Cases 2 and 3, and a 100% concordance in Case 4, in the screening of 50 gene mutations.

Clinicopathological characteristics of patients whose exfoliated cells were used for the *in vitro* survival assay and povidone iodine treatment

The patient characteristics are summarized in Table 3. The histological diagnoses were tubular adenomas in eight patients, intramucosal carcinomas in five patients, and carcinomas with submucosal invasions in two patients. *En bloc* resection was successful in all the patients. Thirteen of the 15 patients (86%) underwent complete resections, one had a venous invasion and the other a positive horizontal resection margin.

Impact of ESDs and tumor characteristics on viability and viable cell counts

The correlation between tumor size, ESD time, viable cell counts, and viability of the exfoliated tumor cells was assessed. The median viable cell counts and median viability of the exfoliated cells in the intraluminal lavage samples at the end of the ESD were 4.9×10^5 cells/ml (2.3×10^5–1.1×10^6) and 24% (9–33%), respectively. With respect to the relevance of the viability and the ESD, the viable cell counts showed a positive relationship with the tumor maximum diameters ($r = 0.464$) and a weak relationship with the ESD times ($r = 0.229$) (Fig. 1a, 1b). However, the viability was not related significantly to the tumor diameters or the ESD times ($r = -0.136$ and $r = -0.179$, respectively) (Fig. 1c, 1d). The correlation between tumor characteristics, viable cell counts, and viability of the exfoliated tumor cells was also examined. With respect to the relevance of viability and tumor characteristics, the viable cell counts and viability were not associated significantly with histological types, cytology statuses, and tumor locations (Fig. 2).

Impact of time course and povidone-iodine on the viability and viable cell counts

In the relationship between viability and time course, the viable cell counts did not show a significant different at 0, 3, 6, and 12 h after the ESD (median viable cell counts: 4.9×10^5, 3.0×10^5, 2.9×10^5, 3.6×10^5, respectively) (Fig. 3a). The median viability of the exfoliated cells in the intraluminal lavage samples was not different in the course of 0–12 h after the ESD. They were 24% (9 – 33%), 24% (8 – 50%), 16% (8 – 48%) and 21% (6 – 46%) at 0, 3, 6, 12 h after the ESD, respectively ($P = 0.872$) (Fig. 3b). Next, the cytotoxic effects of povidone-iodine were examined at different concentrations. Viable cell counts were

found to decrease significantly with the use of 0.5%, 1.0%, and 2.0% povidone-iodine solutions compared with the control (median viable cell counts: 2.2×10^5, 1.4×10^5, 1.1×10^5, and 4.9×10^5, respectively) (Fig. 4a). The median viability of the exfoliated cells in the intraluminal lavage samples at the end of the ESD with 0%, 0.5%, 1.0%, and 2.0% povidone-iodine solutions were 24% (9 – 33%), 20% (10 – 33%), 17% (1 – 30%) and 11% (0 – 16%), respectively (Fig. 4b). Povidone-iodine (2.0%) reduced the cell viability in the samples, significantly, compared with the controls and lower concentrations of povidone-iodine.

Discussion

This study revealed the genetic background of tumors and the exfoliated tumor cells. The NGS analysis revealed that tumors targeted for ESDs had already acquired mutations in many genes involved in cell proliferation, angiogenesis, and invasion, including the *APC*, *RAS*, *TP53*, *KIT*, *FBXW7*, *RET*, *PTEN*, *ATM*, *PDGFRA*, *KDR*, *FLT3*, and *SMAD4* genes. Mutations in these genes are closely related to the ability of tumor cells to implant. In addition, the exfoliated tumor cells and the primary tumor had not only mutations in the same gene, but also mutations at the same locus and type in the gene. Although there were a small number of genes analyzed, this study confirmed that the exfoliated tumor cells were derived genetically from the primary tumor and acquired genetic mutations that favored survival and subsequent growth.

However, even if the exfoliated tumor cells acquired a large number of gene mutations, they would not grow into recurrent tumors if they were not alive. The second aim of this study was to quantify the live exfoliated cells after the ESD and determine how long they could survive. The results suggested that a large number of living cells (1.7×10^5 to

7.9×10^6) were present in the lavage samples after the ESD. While the endoscopist does not incise the tumor itself during the ESD, the normal mucosa with a free margin of a few millimeters from the tumor is incised. Therefore, most of the exfoliated cells are considered to be normal cells. However, as the tumor itself exists in the lumen in an exposed position, it is assumed that the its cells are exfoliated from the primary tumor by physical manipulation. In this study, the tumor cell detection rate by cytology in the lavage sample after the ESD was 50%. A previous study showed a positive rate of tumor cells in the lavage sample of between 25% to 88% [16]. As the confirmation of tumor cells in the lavage sample relied on a morphological diagnosis using cytology, factors affecting the cytology, such as the specimen collection methods and contamination, may have resulted in a lack of reproducibility in the positive cytology rates. However, in any case, there was no doubt that a certain number of tumor cells were present in the lavage sample after the ESD. Notably, the viable exfoliated cells had the ability to survive for more than 12 hours, and the number and viability of exfoliated cells did not decrease over time for 12 hours. These results suggested that the ESD produced live exfoliated tumor cells and that these cells could survive after exfoliation from the primary tumors.

The exfoliated cell numbers correlated with the tumor sizes, but not with tumor localization or malignancies. The primary tumor sizes of the reported cases of local recurrences due to implantation after the ESDs were large, ranging from 65 to 155 mm [13-15]. It should be noted that Nakano et al. reported the recurrence of adenoma after a curative ESD for rectal adenocarcinoma *in situ* [15]. This suggested that the risk of recurrences due to implantation of exfoliated cells should not be underestimated in ESDs for large tumors, even in the absence of T1 cancer. Our results obtained using the NGS

also suggested that there was no definitive difference in gene mutations between Tis and T1 cancers.

The final study aim was to investigate how to treat exfoliated tumor cells after ESDs. In rectal cancer surgery, rectal washouts have been proposed to prevent tumor cell implantation [22, 23]. Maeda et al. reported that the irrigation volumes determined the efficacy of the rectal washouts and that 1500 mL of saline lavage was required to eliminate cancer cells in patients with tumors below the peritoneal reflection [23]. A previous study by these authors, showed that an intraluminal lavage of 1000 mL or more can remove tumor cells exfoliated during ESD [17]. Although 1500 mL of rectal washout in rectal cancer surgery can be performed easily in a short time, endoscopic lavages of 1000 mL or more after ESD are time-consuming. Therefore, in this study, the use of cytotoxic agents to develop an easier method to reduce the viability of the exfoliated cells was considered. *In vitro* drug cytotoxicity testing reported that povidone-iodine and chlorhexidine-cetrimide were quick-acting and the most cytotoxic to tumor cells [24]. Basha et al. reported that washing out with a relatively high concentration povidone-iodine solution (e.g. more than 5%) may be more useful in killing viable exfoliated tumor cells during surgery for colorectal cancer by decreasing the cytotoxicity caused by the presence of proteins, red blood cells or feces [25]. Banich et al. reported that there was no apparent systemic toxicity when using 500 to 1000 mL of a 10% povidone-iodine solution [26]. In the current study, the addition of povidone-iodine solution at a concentration as low as 2.0% was found to significantly reduce cell viability. Although this study was an *in vitro* treatment, the same effect can reasonably be expected in the clinical setting, as cells obtained from clinical samples were treated with iodine solution at a concentration that can be used in the clinical practice. Thus, endoscopic lavages with

2.0% povidone-iodine saline after colorectal ESDs can reduce the quantity of exfoliated tumor cells safely and effectively and prevent local recurrences caused by tumor cell implantations.

This study had several limitations. First, this study included a small number of patients from a single institution. Larger multicenter randomized studies will be necessary to examine the effects of intraluminal lavage on the local recurrence rate. Second, the exfoliated cells from the intraluminal lavage samples contained various cell types, which may have led to inaccurate free tumor cell counts. Third, this was an *in vitro* evaluation of the viability of exfoliated cells in povidone-iodine solutions and thus the results may be different in *in vivo* intraluminal lavages. Despite these limitations, an intraluminal lavage with povidone-iodine after a colorectal ESD is a brief procedure, and we suggest that the results of this study may contribute to the reduction of local recurrences in colorectal ESDs.

In conclusion, this study showed for the first time, that a large number of tumor cells that acquired survival-favorable gene mutations were exfoliated during colorectal ESDs and could survive for a significant time. Moreover, a lavage using a solution of 2.0% povidone-iodine could be an effective treatment against such cells.

Conflict of interest statement: Takayuki Nakamoto and other co-authors have no conflict of interest.

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Figure Legends

Fig.1 Relationship between the viability and ESD (endoscopic submucosal dissection). a) The tumor maximum diameter shows a positive relationship with the viable cell counts (r = 0.464), b) The time required for the ESD shows a weak relationship with the viable cell counts (r = 0.229). c, d) The tumor maximum diameter (r = -0.136) and time required for the ESD (r = -0.179) show no significant relationship with viability.

Fig.2 The relation between the viability and tumor characteristics.
Histological types, cytology statuses and tumor locations show no significant relationship with the viable cell counts and viability.

Fig.3 Viability assessment after ESD (endoscopic submucosal dissection).
The viable cell counts and viability show no differences at 0, 3, 6 and 12 h after the ESD.

Fig.4 Viability assessment after the addition of povidone-iodine solutions.
The viable cell counts show a significant decrease with the 0.5%, 1.0% and 2.0% povidone-iodine solutions compared with the control. A 2.0% povidone-iodine solution shows a significant reduction in the cell viability of the samples compared with the control and low concentrations.

Table 1 List of cancer-related genes covered by next-generation sequencing panel.

AmpliSeqTM for Illumina Cancer HotSpot Panel version 2
ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4
EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2
JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA
PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL

The Ampliseq Cancer Hotspot Panel version 2.0 is a targeted resequencing assay for researching somatic mutations across the hotspot regions of the listed 50 genes.

Table 2 Evaluation of gene mutations between resected tumors and exfoliated tumor cells after ESD

		Case		1		2		3		4	
		Tumor depth		T1		Tis		Tis		T1	
		Tumor size (mm)		33		35		25		28	
Gene	Chr	Loci	Ref	Resected tumor	Exfoliated cells	Resected tumor	Exfoliated cells	Resected tumor	Exfoliated cells	Resected tumor	Exfoliated cells
ALK	2	29432625	C							C/A	C/A
	2	29443617	C							C/G	C/G
ERBB4	2	212578373	-		-/A						
	2	212578391	AAAG		AG/AGAA		AG/AGAA				
MLH1	3	37067240	T					A/A	A/A		
FGFR3	4	1807894	G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
PDGFRA	4	55141054	CAG	CGG/CGG	CGG/CGG	CGG/CGG	CGG/CGG	CGG/CGG	CGG/CGG	CGG/CGG	CGG/CGG
KIT	4	55593464	A	A/C	A/C						
KDR	4	55972974	T	A/A	A/A	T/A	T/A	T/A	T/A	T/A	T/A
FBXW7	4	153249384	CG			CG/CA	CG/CG				
APC	5	112175672	GAAAAG							GAAAAG/ GAAA	GAAAAG/ GAAA
	5	112175770	GG	AG/AG	AG/AG	AG/AG	AG/AG	AG/AG	AG/AG	AG/AG	AG/AG
CSF1R	5	149433596	TG	GA/GA	GA/GA	GA/GA	GA/GA	GA/GA	GA/GA	GA/GA	GA/GA
SMO	7	128845088	A			A/G	A/G				
ABL1	9	133750318	C								

(continued)

		Case		1		2		3		4	
		Tumor depth		T1		Tis		Tis		T1	
		Tumor size (mm)		33		35		25		28	
Gene	Chr	Loci	Ref	Resected tumor	Exfoliated cells	Resected tumor	Exfoliated cells	Resected tumor	Exfoliated cells	Resected tumor	Exfoliated cells
NOTCH1	9	139399409	CAC			CAC/-	-/-				
RET	10	43613843	G	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T
	10	43615572	-A					A/T			
	10	43615633	C			C/G	C/G				
PTEN	10	89624218	C					C/G	C/G		
HRAS	11	534242	A							A/G	A/G
ATM	11	108117835	T			T/C	T/C				
KRAS	12	25398280	GCCACC							GCCACC/ GCCACA	GCCACC/ GCCACA
FLT3	13	28610183	A	A/G	A/G	G/G	G/G	G/G	G/G	G/G	G/G
RB1	13	49033902	TC					TC/CC			
TP53	17	7578208	TG			TG/TG	TG/CG				
	17	7578270	ATG							ATG/AAG	ATG/AAG
	17	7578384	GCAGCGCTCA TGGTGGGG			GCAGCGCTCA TGGTGGGG/-					
ERBB2	17	37881388	A					A/G			
SMAD4	18	48586344	C			C/T	C/T				
	18	48604749	G	G/T	G/T						

Chr, chromosome; Ref, reference; SNV, single nucleotide variants; INS, insertion; DEL, deletion; MNV, multi-nucleotide variants; UTR, untranslated region

Table 3 Clinical and histological characteristics in enrolled cases

Characteristics			
Median age (yrs, range)		69	(49-87)
Sex	Male	9	(60.0%)
	Female	6	(40.0%)
Location	Cecum	1	(6.7%)
	Ascending colon	2	(13.3%)
	Transverse colon	1	(6.7%)
	Descending colon	2	(13.3%)
	Sigmoid colon	2	(13.3%)
	Rectum	7	(46.7%)
Tumor shape	0-Is	4	(26.7%)
	0-IIa	9	(60.0%)
	0-IIc	2	(13.3%)
Median maximum tumor diameter (mm, range)		27	(16-65)
Median ESD time (min, range)		76	(24-127)
Histological diagnosis	LGA	1	(6.7%)
	HGA	7	(46.7%)
	M	5	(33.3%)
	SM	2	(13.3%)
Cytology	Positive	7	(50.0%)
	Negative	7	(50.0%)
	Not performed	1	
En bloc resection		15	(100%)
Curative resection		13	(86.6%)
Vascular invasion		0	
Lymphatic invasion		1	(6.7%)
Margin status	Negative	14	(93.3%)
	Positive	1	(6.7%)

ESD, endoscopic submucosal dissection; LGA, tubular adenoma with low grade atypia; HGA, tubular adenoma with high grade atypia; M, intramucosal carcinoma; SM, carcinoma with submucosal invasion