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Colonic stent-induced mechanical compression may suppress cancer cell proliferation in malignant large bowel obstruction

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Abstract

Background The short-term safety and efficacy of insertion of a self-expandable metallic colonic stent (SEMS) followed by elective surgery, "bridge to surgery (BTS)", for malignant large bowel obstruction (MLBO) have been well described; however, the influence on long-term oncological outcomes is unclear. The aim of this study was to evaluate changes in oncological characteristics in colorectal cancer (CRC) tissues after SEMS insertion, focusing on growth factors, cell cycle and apoptosis.

Methods From January 2013 to September 2014, a total of 25 patients with MLBO who underwent BTS at our single institution were retrospectively included. Paired CRC tissue samples before (endoscopic biopsy) and after SEMS insertion (surgically resected) were collected from each patient. EGFR, VEGF, Ki-67, $p27^{kip1}$ and TUNEL expression were determined by immunohistochemistry.

Results No clinical or subclinical perforations evaluated by mechanical ulceration pathologically were observed. Epithelial exfoliation, tumour necrosis, infiltration of inflammatory cells and fibrosis were observed in SEMS-inserted surgically-resected specimens. Overall, 84% (21/25) and 60% (15/25) of patients exhibited no change or a decrease in staining category, respectively, for EGFR and VEGF expression after SEMS insertion. A significant decrease in Ki-67 expression was observed in surgically-resected specimens compared with endoscopic biopsy specimens ($P < 0.01$). The upstream cell cycle inhibitor, $p27^{kip1}$, was significantly increased after SEMS insertion ($P = 0.049$).

Conclusions Although the long-term safety of BTS should be determined in a future clinical trial, mechanical compression by SEMS may suppress cancer cell proliferation and this result could provide some insights into the issue.

Keywords Colorectal cancer · Obstruction · Self-expandable metallic colonic stent · Bridge to surgery · Mechanical compression

It is estimated that colorectal cancer (CRC) was the most commonly diagnosed malignancy and the second leading cause of cancer-related death in Japan in 2015. Previous reports demonstrated that 8–29% of patients with primary CRC present with an emergent obstruction at the time of diagnosis, which in turn accounts for 85% of colonic emergencies [1–3]. In the past, malignant large bowel obstruction (MLBO), a life-threatening oncological trauma, has been managed by emergency surgical decompression. It frequently requires temporary or permanent colostomy, with high mortality and morbidity rates (7–22% and 30–60%, respectively) [2, 4, 5]. These high morbidity and mortality rates are because of patients' poor general condition and unprepared intestine [2, 4]. Colostomy impairs quality of life, and only 60% of patients who undergo Hartmann's procedure also undergo colostomy closure [6].

Since the first report by Dohmoto in 1991 from Japan, which demonstrated the effective placement of a self-expandable metallic colonic stent (SEMS) for the relief of colonic obstruction, insertion of SEMS is currently recognized as an optimal alternative intervention for

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decompression of MLBO [7]. Although it was initially proposed as a palliative treatment for MLBO, stenting is also increasingly being used as a 'bridge to surgery' (BTS) in potentially curable disease to convert high risk emergency surgery to elective one-stage radical surgery with waiting times optimized for a patient's general condition [8, 9]. Previous systematic reviews have shown more favourable short-term outcomes of the BTS strategy than emergency surgery alone in terms of morbidity, stoma creation, primary anastomosis and length of postoperative hospital stay [10–12].

The major concern of the BTS strategy is the influence on long-term oncological outcomes. Some studies demonstrated worse survival and higher local recurrence rates in BTS-treated patients compared with emergency surgery, however, our recent meta-analysis demonstrated relatively equivalent oncological outcomes [13–15]. Currently, data from reliable randomized controlled trials evaluating this issue are not available. Theoretically, perforation and mechanical manipulation induced by SEMS could have a negative impact on oncological outcomes. In terms of perforation, previous studies demonstrated that SEMS-induced intestinal perforation was a strong risk factor for local recurrence and dissemination [16, 17]. In contrast, some pilot studies evaluated the histological findings after SEMS insertion in MLBO patients [18–21], however, no studies investigating the oncological influences, including tumour growth and progression, of SEMS-induced mechanical manipulation have been reported to date. Thus, this concern is currently at the centre of a controversy.

The aim of this in vivo retrospective study was to evaluate changes in CRC tissues after SEMS insertion, focusing on growth factors, cell cycle and apoptosis. The results of this study could provide insights into this unresolved issue.

Materials and methods

Study patients

This single-institutional retrospective study was approved by the Institutional Review Board of the Nippon Medical School Chiba Hokusoh Hospital (Chiba, Japan) (approval No. 514) and was performed in accordance with the principles contained within the Declaration of Helsinki. Written informed consent was obtained from all participants. From January 2013 to September 2014, a total of 25 patients with MLBO resulting from primary CRC who underwent the BTS strategy (SEMS insertion followed by primary tumour resection) in our department were included. A diagnosis of MLBO was made according to the following characteristics on admission: (1) clinical symptoms of abdominal distention, pain and constipation, (2) CT scan findings of dilatation of the colon without intestinal perforation and (3) endoscopic and pathological confirmation of an obstructive primary colorectal tumour.

The patients' demographic baseline and surgical variables were retrospectively collected.

Endoscopic stenting and surgical procedures

Urgent colonoscopy for MLBO was performed to evaluate the obstructing tumour and to obtain a biopsy specimen. Water-soluble contrast medium via endoscopy was concomitantly injected to confirm the length and morphology of the tumour. Under fluoroscopic and endoscopic guidance, a guide wire (0.035 inches in diameter) was introduced through the tumour beyond the point of the obstruction and proximally to the distended colon. The SEMS, WallFlex Colonic Stent (Boston Scientific, Marlborough, MA) or Niti-S Colonic Stent (Taewoong Medical Inc., Gimpo-si, Korea), was developed over the wire. All SEMSs were bare (uncovered). Radiological examination was performed the day after SEMS insertion to rule out intestinal perforation and confirm the position and expansion of the SEMS. All endoscopic SEMS insertion procedures were performed by a single expert physician (A.M.).

After the waiting period from SEMS insertion, various surgical procedures were performed to resect the primary lesions of enrolled patients. All of the included patients underwent successful SEMS insertion with biopsy specimen extraction and achieved sufficient intestinal decompression followed by elective surgery. All surgeries were performed by board-certified colorectal surgeons with equivalent experience in the surgical treatment of CRC.

Pathological examination

Fresh cancer tissue obtained by endoscopic biopsy and surgical resection were fixed in 10% neutral buffered formalin for 24 h. Consecutive 4-μm tissue sections were cut from paraffin-embedded blocks. Considering that the aim of this study was to investigate the alterations in oncological characteristics after SEMS insertion, and that these characteristics are heterogeneous in CRC, the examined region of the endoscopic biopsy and paired surgically-resected specimen from each patient should be consistent, which makes it possible to evaluate the direct influence of mechanical manipulation of SEMS. The tissue sections from the anal-sided round wall of the tumour, which is routinely obtained by endoscopic biopsy, of the surgically-resected specimens were provided for further experiments.

Routine haematoxylin and eosin (H&E) staining was performed to evaluate the histopathological characteristics of the tumours. Immunohistochemical staining (IHC) according to the avidin biotin procedure was performed using the following primary antibodies: anti-VEGF (IBL, Gunma,

Japan), anti-EGFR (EGFR pharmDx kit; DAKO, Carpinteria, CA), anti-Ki-67 (DAKO) and anti-p27^{kip1} (DAKO). In each analysis, CRC samples previously shown to stain with these antibodies were used as positive controls. Phosphate-buffered saline in place of the primary antibody was used as a negative control. To detect apoptotic cancer cells, TUNEL staining using a specific kit (Roche Life Science, Mannheim, Germany) was performed.

Assessment of immunohistochemical staining

Cytoplasmic immunoreactivity for VEGF was evaluated according to the extent of staining, with staining intensities scored as: 0, no cells stained; 1, less than 10% of cell stained; 2, 11–50% of cells stained; 3, more than 50% of cells stained [22]. EGFR expression was shown as membranous staining of cancer cells and the intensity of the staining was categorized as follows: IHC negative (no staining), IHC 1+ (incomplete circumferential staining: weak), IHC 2+ (complete circumferential staining: moderate) and IHC 3+ (complete strong circumferential staining: strong) [23]. For nuclear Ki-67, p27 ^{kip1} expression, and TUNEL staining, the percentage of positive cells from at least 1000 cancer cells from three randomly-selected fields of vision using a high power lens (×400) were calculated [24]. The slides were reviewed by pathologists, who had no prior knowledge of the clinical characteristics of each patient. However, complete blindness of the slides was impossible, because slides derived from endoscopic biopsy or surgically-resected samples are easily distinguishable by their morphology.

Statistical analysis

Continuous data of Ki-67, p27 ^{kip1}, and TUNEL staining are expressed as mean ± standard deviation. The two-tailed Student's *t* test was used to compare continuous variables. A *P* value of <0.05 was considered statistically significant. The scores of VEGF and EGFR staining were categorized by the alterations in the staining of the surgically-resected sample compared with the endoscopic biopsy sample in each patient, as follows: up (increased), no change and down (decreased).

All statistical analyses were performed with R, version 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Patients' characteristics

The clinical characteristics and SEMS-related variables of the 25 enrolled patients are shown in Table 1. Fourteen (56%) patients were male and 11 (44%) were female. The mean patient age was 64 years. The tumours of six patients (24%) were located in the right-sided colon. The majority of inserted SEMSs were Niti-S (22; 88%), 22 mm in diameter (24; 96%) and 6 cm in length (19; 76%). Clinical success of intestinal decompression was achieved in all patients (25; 100%) without any SEMS-related complications including clinical perforation. The mean duration from SEMS insertion to elective surgery was 15.6 days.

Table 1 Clinical characteristics and SEMS-related variables of enrolled patients ($n=25$)

Gender (male/female)	14 (56)/11 (44)
Age (years ± SD)	64 ± 11
Tumour location	
Right/left-sided	6 (24)/19 (76)
Cecum	0 (0)
Ascending	4 (16)
Transverse	2 (8)
Descending	2 (8)
Sigmoid	6 (24)
Rectum	9 (36)
Size of tumour (mm ± SD)	66 ± 18
Type of SEMS	
WallFlex	3 (12)
Niti-S	22 (88)
Diameter of SEMS (mm)	
22/18	24 (96)/1 (4)
Length of SEMS (cm)	
10/8/6	3 (12)/3 (12)/19 (76)
Clinical success	25 (100)
Perforation	0 (0)
Interval form SEMS to surgery (days ± SD)	15.6 ± 7
Surgical procedures	
Ileocecal resection	3 (12)
Right hemicolectomy	2 (8)
Partial colectomy	4 (16)
Sigmoidectomy	7 (28)
Rectal resection	9 (36)

Values are expressed as mean ± standard deviation (SD)

SEMS self-expandable metallic stent

The pathological characteristics of the enrolled patients are shown in Table 2. All 25 patients were diagnosed with adenocarcinoma, and most (22; 88%) were well and moderately differentiated. The depth of all tumours was beyond the muscularis propia. Fifteen patients (60%) were T3 and ten patients (40%) were T4. The majority of tumours presented lymphatic and venous invasion (24; 96% and 21; 84%, respectively). Perineural invasion was identified in 15 patients (60%). SEMS-induced mechanical ulceration reached the submucosa in 7 (28%) patients, muscularis propia in 17 (68%) and subserosa in 1 (4%) patient. That is,

Table 2 Pathological variables of the enrolled patients ($n=25$)

Histology	
Well/mod/por	6 (24)/16 (64)/3 (12)
Depth of tumour (UICC)	
T3/T4a/T4b	15(60)/6 (24)/4 (16)
Lymphatic invasion (positive)	24 (96)
Venous invasion (positive)	21 (84)
Perineural invasion (positive)	15 (60)
Stage (UICC)	
II/III/IV	6 (24)/12 (48)/7 (28)
Depth of SEMS-induced ulceration	
Submucosa	7 (28)
Muscularis propia	17 (68)
Subserosa	1 (4)

no subclinical perforation was observed pathologically. The pathological findings of SEMS-inserted surgically-resected specimens included epithelial exfoliation, tumour necrosis, infiltration of inflammatory cells and fibrosis (Fig. 1A, B).

SEMS-induced changes in oncological molecular markers

Changes in the oncological characteristics of CRC following SEMS-induced mechanical manipulation were evaluated by comparison with the immunohistochemical expression levels of growth factors, cell cycle-related proteins, and apoptosis factors in paired endoscopic biopsy and surgically-resected specimens. Representative images of immunohistochemical staining for membranous EGFR, cytoplasmic VEGF, nuclear Ki-67 and nuclear $p27^{kip1}$ are shown in Fig. 2A–D. EGFR expression demonstrated no significant difference between the paired samples ($P=0.057$). However, only four patients (16%) exhibited an increase in staining category, and the majority (21; 84%) of patients showed no change or a decrease in category after SEMS insertion. Regarding VEGF expression, there was no significant difference in staining category between the paired samples ($P=0.244$). While 15 patients (60%) demonstrated no change or decrease in category, 10 patients (40%) showed an increase (Table 3).

A significant decrease in Ki-67 expression was observed in surgically-resected specimens compared with paired endoscopic biopsy specimens ($34.0\pm5.9\%$ and $49.4\pm7.6\%$, respectively, $P<0.01$), which may suggest that SEMS suppressed cancer cell proliferation (Fig. 3A). Next, to explore the mechanism by which there was a significant suppression of Ki-67 expression after SEMS insertion, we investigated the upstream cell cycle inhibitor, $p27^{kip1}$, before and after insertion. Expression was significantly increased in surgically-resected specimens compared with endoscopic biopsy specimens ($46.7\pm16.2\%$ and $37.0\pm16.4\%$, respectively, $P=0.049$) (Fig. 3B). With regard to the apoptosis of cancer cells, only sparse positive TUNEL staining was observed in endoscopic biopsy specimens and no significant alteration in TUNEL staining positivity was seen in surgically-resected specimens (data not shown).

Discussion

Regardless of the widespread clinical use of SEMS for MLBO, reliable data on its influence on long-term oncological outcomes is lacking and, therefore the issue is not yet resolved. It is believed that SEMS introduction for MLBO could induce perforation and mechanical manipulation of the tumour, which, in turn, can have a negative oncological impact, including peritoneal dissemination, tumour progression and metastasis. In contrast to SEMS-induced colonic perforation, with several studies demonstrating significantly worse survival through tumour spreading into the peritoneal

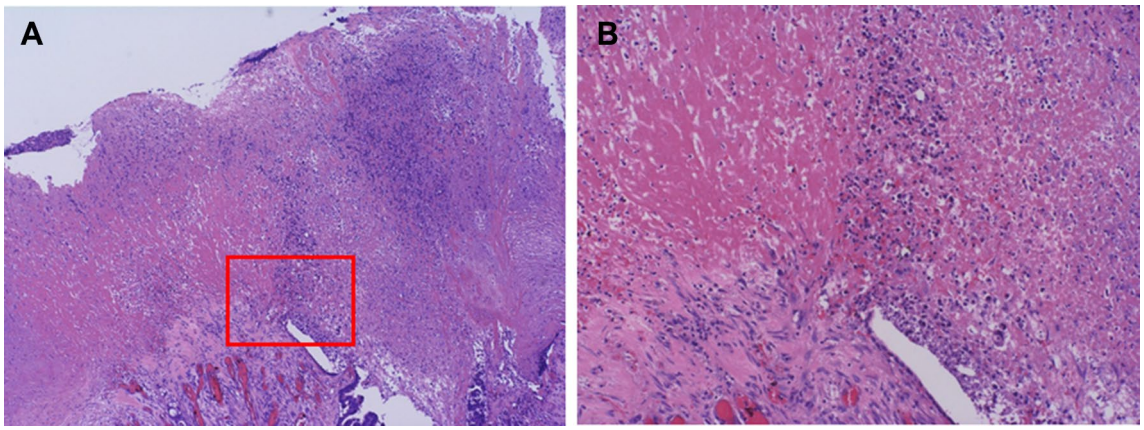

Fig. 1 Representative haematoxylin and eosin staining in surgically-resected colorectal cancer tissue after self-expandable metallic stent insertion. Original magnification ×200 (**A**), ×400 (**B** squared area **A** is enlarged)

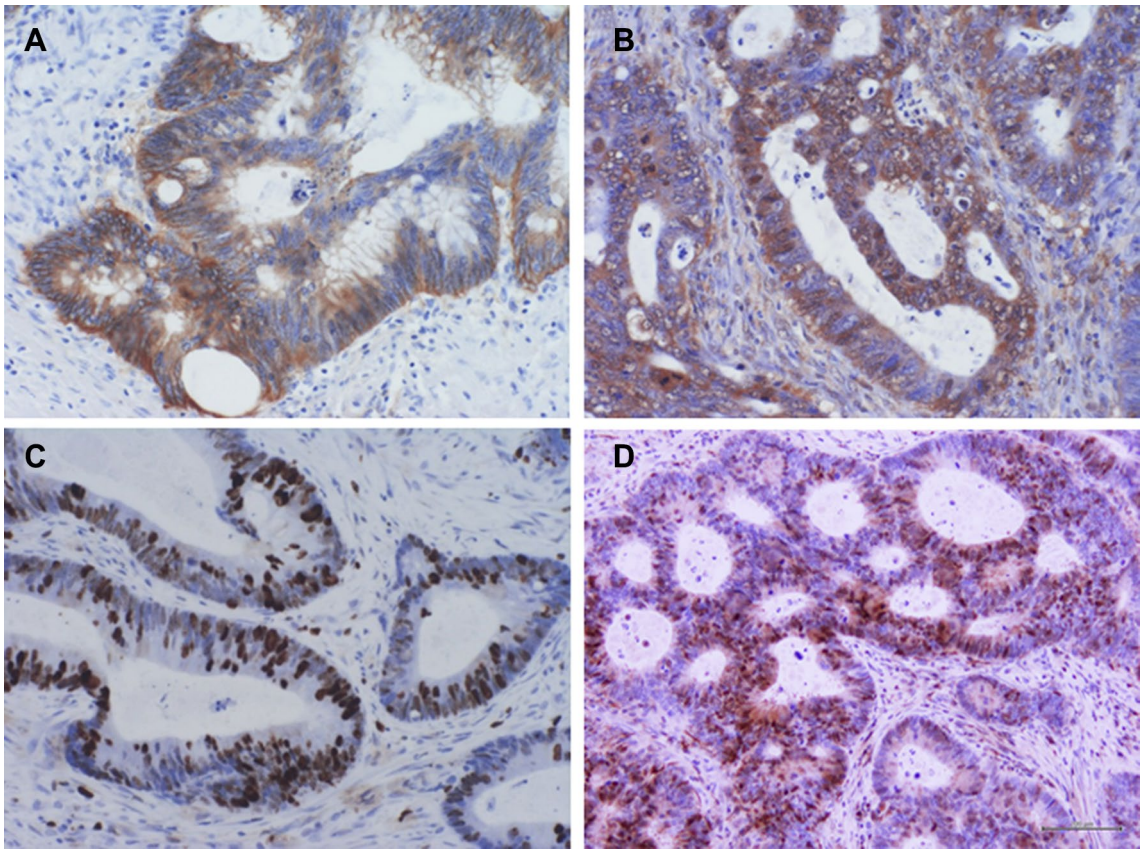

Fig. 2 Representative immunohistochemical staining of **A** EGFR, **B** VEGF, **C** Ki-67, and **D** $p27^{kip1}$ expression. Original magnification × 400

Table 3 SEMS-induced changes in growth factor expression ($n = 25$)

	Before SEMS	After SEMS	*P* value
EGFR			
Category 0	12 (48)	14 (56)	0.057
1+	4 (16)	9 (36)	
2+	9 (36)	2 (8)	
3+	0 (0)	1 (4)	
Alteration			
Up		4 (16)	
No change		13 (52)	
Down		8 (32)	
VEGF			
Category 0	0 (0)	0 (0)	0.244
1+	14 (56)	8 (32)	
2+	10 (40)	15 (60)	
3+	1 (4)	2 (8)	
Alteration			
Up		10 (40)	
No change		12 (48)	
Down		3 (12)	

cavity [16, 17], the oncological influence on cancer tissue of SEMS-induced mechanical manipulation has not been investigated thus far. Previously, a small number of studies assessed the pathological appearance of surgically-resected SEMS-inserted specimens, and revealed characteristics such as tumour indentation, fissuring ulceration and inflammatory changes mimicking inflammatory bowel diseases [19–21]. However, how these SEMS-induced pathological changes affect malignant tumour potential is unknown. In contrast, our study design comparing oncological molecular markers before and after SEMS insertion in each patient seems to be reasonable to assess the direct influence of SEMS on oncological characteristics. On reflection, while SEMS is applied to a variety of clinical settings including malignant biliary, oesophagus, gastric and duodenal obstructions, surgical resection after SEMS insertion (BTS) is clinically accepted only in MLBO. The use of human CRC samples would be the only and the best way to explore this question.

Ki-67 protein is present in the nuclei of cells during mitosis and is involved in the regulation of the cell cycle [24]. Its expression is used as a marker of cell proliferation, and it has been recognized as an independent prognostic marker in various cancers, including CRC [24–28]. Surprisingly, mechanical manipulation by SEMS resulted in a significant

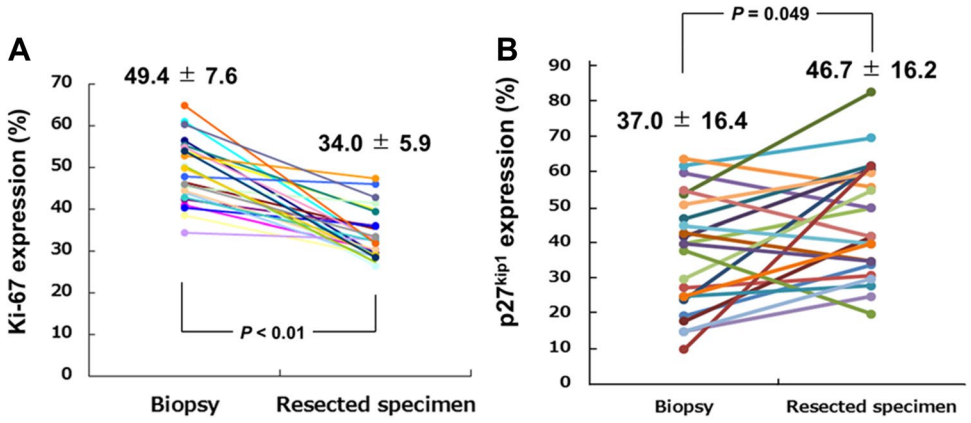

Fig. 3 Self-expandable metallic colonic stent-induced alterations of **A** Ki-67 and **B** $p27^{kip1}$ expression. Values are expressed as mean ± standard deviation

decrease in Ki-67 expression. The mechanical manipulation occurring in constrained environments, consistent with a tumour and the stroma, entails a competition for space. The pathways of communication between a tumour and its microenvironment are diverse, but they can be broadly separated into biochemical and mechanical signals. The biochemical signals have been widely studied in the past [29, 30], however, the mechanical signals are just beginning to be investigated. Recently, several in vitro studies have shown the effect of compressive stress applied on a tumour model system, called a multicellular spheroid (MCS). MCSs are three-dimensional cellular aggregates that remarkably mimic the relevant in vivo physiological gradients of mitogens, oxygen or glucose and have been extensively used as model system for the study of drug delivery, cell proliferation, invasion and angiogenesis [31]. Previous studies have revealed that a compressive stress applied on MCSs grown from a colon carcinoma cell line drastically reduce their proliferation rate and that this reduction is associated with a decrease in cell division rather than to an increase in cell apoptosis [32, 33]. Delarue et al. [34] reported in their consecutive in vitro studies that a compressive stress inhibited the proliferation of colon cancer cells, which were blocked in G1 phase of the cell cycle, through overexpression of the cyclin-dependent kinase inhibitor $p27^{kip1}$. Reduced $p27^{kip1}$ expression is prevalent in a wide range of human cancers including CRC, and its decreased expression is also correlated with more advanced disease stage and poorer oncological outcomes in various cancers [35, 36]. Thus, we investigated alterations of $p27^{kip1}$ expression before and after SEMS insertion to explore the mechanism of reduction of Ki-67 expression. Resected specimens after SEMS insertion showed a significant upregulation of $p27^{kip1}$ expression compared with biopsy specimens, as expected. Furthermore, apoptosis of cancer cells was not induced after SEMS insertion, as previously reported [32, 33].

EGFR is a transmembrane glycoprotein with an intracellular tyrosine kinase domain. Binding of ligands to the EGFR promotes tumour growth and progression by controlling transcription, cell cycle progression, apoptosis and differentiation through the downstream signal pathway [23]. Several studies demonstrated that EGFR overexpression, as assessed by IHC, is associated with poor prognosis in advanced CRC [37, 38]. However, a recent retrospective analysis of two large prospective studies indicated that EGFR IHC could not be predictive for the response to anti-EGFR antibody treatment in metastatic CRC [39]. The reported positivity of EGFR IHC using the US FDA-approved EGFR pharm Dx kit is 25–85% [37]. Thus, a finding of 52% of EGFR-positive endoscopic biopsy specimens in our study seemed to be acceptable. Although it did not reach statistical significance ($P = 0.057$), the categorical distribution before and after SEMS insertion showed a downward trend; 32% of patients had a decreased category and a total of 84% of patients did not show an increase in EGFR expression after SEMS insertion. Interestingly, it was reported that there is a close inverse relationship between $p27^{kip1}$ and EGFR expression in human CRC specimens, and $p27^{kip1}$ was found to negatively regulate EGFR expression via the JNK (c-Jun N-terminal kinase)/c-Jun transcription factor [40].

VEGF is a well-known major regulator of angiogenesis and plays an important role in tumour progression. Several studies observed that overexpression is associated with poor prognosis in CRC patients [22, 41]. The current study demonstrated no change or a decrease in expression category in 60% of the patients, but 40% showed an increase. It is conceivable that SEMS-induced mechanical compression of cancer tissue could exposure cancer cells to a hypoxic environment. Hypoxia is known to be a potent inducer of *VEGF* gene expression [42]. *VEGF* is transcriptionally regulated by hypoxia-inducible factor-1 (HIF-1), which translocates to the nucleus following hypoxia-induced stabilization, and activates hypoxia-inducible genes (HRE) in the promoter region of the *VEGF* genes. The hypoxia–HIF–*VEGF* pathway facilitates neovascularization and tumour progression

[43]. Further comprehensive investigations are required to elucidate the SEMS-induced modification of this pathway.

This study has several limitations. First, statistical analyses of differences were limited by the small sample size from a single institution. Second, complete blindness of examined slides before or after SEMS insertion was impossible because of the morphological differences, but the study quality was secured by withholding the study hypothesis from the pathologist. Third, it is unclear as to what extent changes in the assessed molecular markers can affect the oncological outcomes.

Conclusions

The long-term safety of the BTS strategy for MLBO should be determined by a high-quality large-scale clinical trial (for example, the COBRA trial [44] ongoing in Japan). However, the results of this study demonstrated the possibility of CRC cell proliferation suppression resulting from mechanical compression induced by SEMS, and thus our findings could provide some insights into this unresolved issue.

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Compliance with ethical standards

Disclosures Drs. Akihisa Matsuda, Masao Miyashita, Satoshi Matsumoto, Nobuyuki Sakurazawa, Youichi Kawano, Kazuya Yamahatsu, Kumiko Sekiguchi, Marina Yamada, Tsutomu Hatori, and Hiroshi Yoshida have no conflicts of interest or financial ties to disclose.

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