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Clinical Trial Notes

Prospective Feasibility Study to Evaluate Neoadjuvant-synchronous S-1 + RT for Locally Advanced Rectal Cancer: A Multicenter Phase II Trial (UMIN ID: 03396)

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In Western countries, the standard treatment for locally advanced rectal cancer is preoperative chemoradiotherapy followed by total mesorectal excision. However, in Japan, the treatment results without preoperative chemoradiotherapy are by no means inferior; therefore, extrapolation of the results of preoperative treatment in Western countries to Japan is controversial. We consider that survival may be improved by preoperative chemoradiotherapy with new anticancer agents as they are expected not only to decrease the local recurrence rate but also to prevent distant metastases. We are conducting a multicentre Phase II study to evaluate the safety and efficacy of neoadjuvant chemoradiotherapy using S-1 in patients with locally advanced rectal cancer. The primary endpoint is the rate of complete treatment of neoadjuvant chemoradiotherapy. Secondary endpoints are the response rate of neoadjuvant chemoradiotherapy, short-term clinical outcomes, rate of curative resection and pathological evaluation. The short-term clinical outcomes are adverse events of neoadjuvant chemoradiotherapy and surgery-related complications. Thirty-five patients are required for this study.

Key words: rectal cancer – neoadjuvant chemoradiotherapy – S-1

INTRODUCTION

The standard treatment for locally advanced rectal cancer is well known to differ between Japan and Western countries. In Western countries, multimodal therapies such as preoperative short-term intensive radiotherapy or conventional long-term radiotherapy in combination with 5-fluorouracil (5-FU)-based chemotherapy have gained widespread acceptance for the treatment of locally advanced rectal adenocarcinoma (1). These treatments provide improved local control when compared with surgery alone, although only one study has shown a survival benefit (2). The local control benefit of preoperative radiotherapy remains relevant even in the era of total mesorectal excision (TME) (3). The addition of chemotherapy to preoperative conventional long-term radiotherapy (RT) has been demonstrated to be feasible, with enhanced tumoricidal effects (4). In Japan, TME or tumor-specific mesorectal excision followed by adjuvant chemotherapy without preoperative treatment is a standard strategy, and lateral lymph node (LN) dissection is added in patients with lower rectal cancer (5). The results of the surgical treatment without RT in Japan are by no means inferior to those in Western countries that do use RT with surgery. Therefore, extrapolation of the results of preoperative treatment in Western countries to Japan is controversial.

Recently, new anticancer agents have markedly improved the response rate and prognosis of unresectable and recurrent colorectal cancer. Locally advanced rectal cancer may be controlled by the addition of new anticancer agents. In Western countries, new treatment strategies have been tested, including the addition of new cytotoxic drugs and/or

molecular-targeted drugs to fluoropyrimidine-based chemoradiotherapy concurrently or before chemoradiotherapy (6,7). On the other hand, there is a concept that oral chemotherapy has major advantages over intravenously administered treatment in terms of pharmacoeconomic considerations and patient preferences, because oral treatment can be administered on an outpatient basis, thereby reducing the length of patients' hospital stays (8). Over time, the role of oral chemotherapy in the treatment of malignant disease is expected to become increasingly significant. S-1 (TS-1, Taiho Pharmaceutical) is an orally active combination of tegafur (a prodrug that is converted by cells to fluorouracil), gimeracil (an inhibitor of dihydropyrimidine dehydrogenase, which degrades fluorouracil) and oteracil (which inhibits the phosphorylation of fluorouracil in the gastrointestinal tract, thereby reducing the gastrointestinal toxic effects of fluorouracil) in a molar ratio of 1:0.4:1 (9,10). The rate of response to treatment with S-1 alone exceeded around 40% in two Phase II trials involving patients with advanced or recurrent colorectal cancer (11,12). Furthermore, S-1 has been demonstrated to enhance the radiation response of human colon cancer xenografts resistant to 5-FU (13). In 2011, Sadahiro et al. (14) reported that the efficacy of chemoradiotherapy with S-1 seems to be equivalent to the efficacy reported for chemoradiotherapy with capecitabine. However, the dose of S-1 (100 mg/m^2) in our study is different from that of S-1 (80 mg/m^2) in the above-mentioned study. We planned the present study in order to obtain the more excellent efficacy.

We consider that survival may be improved by preoperative treatment with new anticancer agents, S-1 as they are expected to decrease local recurrence due to their effect of bulk reduction, to obtain a high rate of complete treatment of neoadjuvant chemoradiotherapy and to prevent distant metastases.

We conducted our own Phase II study to confirm the safety and efficacy of the chemoradiotherapy using S-1 before surgery. Our administration schedule of S-1 is 100 mg/m^2/day for 5 days, and followed by no administration for 2 days. The total dose of S-1 per week is 500 mg/m^2/week. On the other hand, S-1 at 80 mg/m^2/day is the standard dose used as a single agent for systemic therapy, which gives a total of 560 mg/m^2/week. Because the total dose of S-1 per week in our study (500 mg/m^2) is less than the standard amount per week (560 mg/m^2), Phase I trial has not been conducted.

The institutional review board of each participating center approved the study protocol. This study was registered at the UMIN Clinical Trial Registry as UMIN000003396 (http://www.umin.ac.jp/ctr/index.htm).

PROTOCOL DIGEST OF THE OITA TRIAL

Purpose

To evaluate the feasibility and efficacy of neoadjuvant CRT for locally advanced rectal cancer.

Study Setting

A multi-institutional (17 specialized centers), interventional Phase II trial. This study is registered with UMIN-CTR, number C003396.

Resources

This study was supported by a part of Grants-in-Aid for Clinical Cancer Research from the Japanese Ministry of Health, Labour and Welfare (22-Clinical Cancer-027).

Endpoints

The primary endpoint is the rate of complete treatment of neoadjuvant chemoradiotherapy. Secondary endpoints are the response rates of neoadjuvant chemoradiotherapy, short-term clinical outcomes, rate of curative resection and pathological evaluation. The short-term clinical outcomes are adverse events of neoadjuvant chemoradiotherapy, surgery-related complications. The response rate is evaluated using RECIST, and the adverse events including preoperative chemoradiotherapy and surgical complication are evaluated using CTCAE v4.0.

Eligibility Criteria

Tumors are staged according to the TNM classification system.

Inclusion Criteria

For inclusion in the study, patients must fulfill the following requirements before neoadjuvant chemoradiotherapy: (i) histologically proven rectal carcinoma; (ii) tumor located in the rectum (Ra,Rb,P); (iii) cancer classified as T3–4, N0–3 and M0, according to the TNM classification system; (15) no bowel obstruction; (v) age >20 and <80 years; (vi) sufficient organ function; (vii) no history of gastrointestinal surgery; (viii) no history of chemotherapy or radiotherapy and (ix) provide written informed consent.

Exclusion Criteria

The exclusion criteria are as follows: (i) synchronous or metachronous (within 5 years) malignancy other than carcinoma *in situ*; (ii) critical drug sensitivity to S-1; (iii) severe pulmonary emphysema, interstitial pneumonitis or ischemic heart disease; (15) pregnant or lactating women; (v) severe mental disease; and (vi) continuous systemic steroid therapy.

Treatment Method

For the locally advanced rectal carcinoma, two cycles of neoadjuvant chemotherapy with S-1 (100 mg/m^2 on Days 1–5, 8–12, 22–26 and 29–33) is administered, and

irradiation (total 45 Gy/25 fr, 1.8 Gy/day, on Days 1–5, 8–12, 15–19, 22–26 and 29–33) is performed.

Additional Treatment

Resection of the rectum with D3 lymphadenectomy is performed according to the Japanese Classification of Colorectal Carcinoma (Japanese Society for Cancer of the Colon and Rectum. General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus, 6th edn, 1998 (in Japanese)). Operation is performed during the 4th and 8th week after the end of the neoadjuvant chemoradiation therapy. Proposed operations are anterior resection with or without covering ileostomy and anterior peritoneal resection. When the preoperative and intraoperative findings demonstrate that the lateral LNs metastasis is not suspected, lateral LNs dissection is not performed. The adjuvant chemotherapy is not specified.

Follow-up

Patients are observed by their surgeon every 3–4 months after operation. Blood tests, abdominal computed tomography and plain chest X-ray are carried out at each visit.

Study Design and Statistical Method

This trial is designed to achieve the feasibility and efficacy of neoadjuvant CRT with S-1 for locally advanced rectal cancer in terms of completion rate, efficacy and adverse events of neoadjuvant chemoradiation and curative resection rate. If the feasibility and efficacy of neoadjuvant CRT with S-1 is shown, neoadjuvant CRT with S-1 will be the preferred treatment. The planned sample size is 35 patients, which was calculated by Southwest Oncology Group's two-stage attained design (16) based on a target rate of treatment completion of 90% and a minimum completion rate of 70%, with an a error of 0.05 and b error of 0.15.

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Conflict of interest statement

None declared.

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Multivariate Evaluation of the Technical Difficulties in Performing Laparoscopic Anterior Resection for Rectal Cancer

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Background: Although the laparoscopic approach is accepted for the treatment of colon cancer, its value for low rectal cancer is unknown. We sought to evaluate the technical feasibility of laparoscopic low anterior resection (Lap-AR) by determining short-term clinical outcomes and identifying the corresponding predictive factors.

Methods: A retrospective single-institution study was carried out on 82 patients in whom Lap-AR had been attempted for rectal cancer during the period spanning April 2001 to December 2009. Patient characteristics, operative outcomes, and postoperative morbidities and mortalities were analyzed.

Results: The median operative time and the intraoperative blood loss were 300 minutes and 72.5 g, respectively. Overall morbidity and mortality rates were 11.0% and 0%, respectively. Complications included wound infection (6.1%, n = 5), anastomotic leakage (1.2%, n = 1), ileus (1.2%, n = 1), and pneumonia (2.4%, n = 2). A multivariate analysis indicated that the important risk factor associated with an operative time of > 300 minutes was the T factor, and the risk factor associated with intraoperative blood loss was a body mass index (BMI) of > 25 kg/m^2.

Conclusions: Lap-AR is a technically feasible, safe, and effective method for treating patients with rectal cancer. A BMI > 25 kg/m^2 and the T factor related to operative blood loss and operative time, respectively. Assessment of high BMI and, in particular, advanced tumor depth, should alert surgeons to the increased technical difficulty of Lap-AR.

Key Words: rectal cancer, laparoscopic anterior resection, surgical outcome, risk factor

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Although the laparoscopic approach for the treatment of colon cancer has become increasingly accepted and popular, the role of laparoscopic resection in the treatment of rectal cancer is still controversial. Rectal cancer was excluded from most trials of laparoscopic resection of the large bowel because of the technical complexity of the procedure.[1–7] First, the anatomic position of the rectum makes access more difficult; second, total mesorectal excision (TME) is important for reducing local recurrence; and finally, preservation of the autonomic nerves and the sphincter apparatus are important to maintain bladder control, continence, and sexual function, which represent important aspects of the quality of life after surgery.[8] Laparoscopic surgery for rectal cancer, as with open surgery, is associated with a considerably higher rate of complications than that of colonic surgery, especially if the surgeon does not have sufficient experience in open TME and advanced laparoscopic surgery. However, a similar incidence of complications has been reported for both open and laparoscopic techniques,[9–14] and no differences in complications have been found in meta-analyses.[15,16]

The conventional versus laparoscopic-assisted surgery in patients with colorectal cancer (CLASICC) trial[6] initially reported a greater circumferential margin involvement in rectal cancer treated by laparoscopy (although the difference was not significant) and, at the time, recommended caution when treating rectal cancer by the laparoscopic approach. We retrospectively reviewed the data related to all the laparoscopic low anterior resections (Lap-ARs) performed by 3 experienced colorectal cancer surgeons at our institution with the intent of establishing background data. The objective of this single-institution retrospective study was to evaluate the technical feasibility of Lap-AR by determining short-term clinical outcomes. We also attempted to identify predictive factors related to short-term clinical outcomes of Lap-AR.

PATIENTS AND METHODS

Patient Selection Criteria

We reviewed the medical records of all 82 rectal cancer patients in whom Lap-AR was attempted between April 2001 and December 2009 by 3 well-experienced, board-certified laparoscopic colorectal surgeons (K.Y., M.I., and S.K.) at our institution. Basically, we did not perform preoperative radiotherapy; however, patients who were preoperatively diagnosed to have very advanced cancer with invasion into an adjacent organ underwent preoperative radiotherapy. The tumor location was defined as the distance between the distal margin of the tumor and the anal verge, as measured by barium enema, and classified as Rb rectum (0 to 5 cm), Rb rectum (5.1 to 10 cm), and Rs rectum (10.1 cm). Lap-AR for rectal cancer was performed successfully in all patients. All participating surgeons were personally responsible for obtaining written informed consent from their patients.

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Indications for Lap-AR

The initial indication for Lap-AR was mucosal or submucosal (cT0, 1) rectal cancer. Patients suitable for endoscopic mucosal resection or those with a history of lower abdominal surgery were excluded. Since 2007, however, the criteria have been extended by some investigators to include patients with preoperative cT2-4 N0-3 M0 lesions.

Surgical Procedure

Laparoscopic resection was performed as follows: laparoscopic AR is performed for the treatment of early rectal cancer located just above the dentate line and advanced rectal cancer located > 1 cm above the dentate line. These criteria enable the acquisition of an adequate distal margin after rectal transection. Patients were placed in the Lloyd-Davis position, with the head and the right side of the bed lowered. First, a 12-mm camera port was inserted below the umbilicus using the open method. After the creation of a pneumoperitoneum, 4 working ports were inserted: 5-mm ports in the right and the left upper abdominal quadrants and 12-mm ports in the right and the left lower abdominal quadrants. After mobilization of the left colon, if necessary, mobilization of the splenic flexure was performed; intracorporeal ligation of the inferior mesenteric vessels was performed followed by mobilization of the rectum and the mesorectum. This permitted the performance of the laparoscopic no-touch isolation technique, the so-called "medial-to-lateral" approach and TME, except in the case of midrectal cancer, where a tumor-specific mesorectal excision was performed. After the completion of full mobilization by cutting the peritoneum from the lateral side, intracorporeal transection of the distal bowel was performed with an Endo GIA stapler (Ethicon Endo-Surgery, Cincinnati, OH). Usually, 2 stapler cartridges were intentionally used for complete resection. The bowel was exteriorized through a 4- to 6-cm incision made under a wound protector over the midlower port site and was divided with appropriate proximal bowel, following which the proximal anvil was then placed. The anastomosis was performed by means of the double-stapling technique.

Quantification of Visceral Fat Area

Accumulation of visceral adipose tissue was determined by measuring the area of such tissue at the single level of the umbilicus by computed tomography (CT). Visceral obesity was defined as a visceral fat area (VFA) of 100 cm^2 or more according to the Japan Society for the Study of Obesity.[17] All quantification of VFA for the procedures described above was performed by a single examiner (T.A.). The free software, Scion Image (http://www.scioncorp.com/), was used to measure the area of visceral adipose tissue.

TABLE 1. Clinicopathologic Characteristics of Patients Undergoing Lap-LAR (n = 82)

Variables	Median (Range)	n	%
Age (y)			
≤60	56.5 (38-60)	27	32.9
>60	73 (61-95)	55	37.1
Sex			
Male	—	49	59.8
Female	—	33	40.2
BMI (kg/m^2)			
≤25	21.2 (16-24.5)	65	79.3
>25	26.5 (25.3-30.5)	17	20.7
VFA (cm^2)			
≤100	85.3 (65.0-100)	42	51.2
>100	146.8 (102-266)	40	48.8
Pelvic width (mm)			
≤190	178.2 (145.8-189.0)	41	50
>190	205.0 (193.8-263.3)	41	50
Comorbidity			
None	—	32	39
One or more	—	50	61
Tumor location			
Rs/Ra	—	66	80.5
Rb	—	16	19.5
T factor			
T1/2	—	29	35.4
T3/4	—	53	64.6
Tumor size (mm)			
≤40	27.0 (10-40)	43	52.4
>40	52.5 (42-90)	39	47.6
Lymph node dissection			
D1/2	—	28	34.1
D3	—	54	65.9
Creating ileostomy			
Present	—	6	7.3
Absent	—	76	92.8
Conversion to open			
Present	—	1	1.2
Absent	—	81	98.8
Preoperative radiotherapy			
Present	—	5	6
Absent	—	77	94

BMI indicates body mass index; Lap-AR, laparoscopic low anterior resection; VFA, visceral fat index.

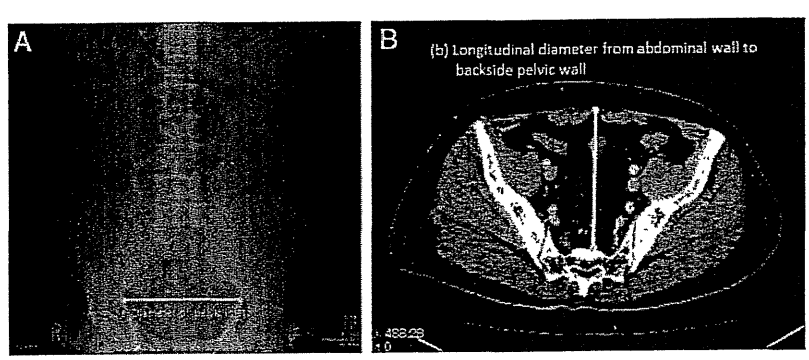

FIGURE 1. A, An abdominal x-ray showing the measurement of the pelvic inlet. B, A computed tomography image showing the measurement of the longitudinal diameter from the abdominal wall to the backside pelvic wall, which corresponds to the location of the pelvic inlet diameter measured in (A).

Definition of Pelvic Width

All patients underwent both abdominal x-ray and abdominopelvic CT with a slice interval of 5 mm. A single observer first measured (a) the diameter of the pelvic inlet on abdominal x-ray (Fig. 1A) and then measured (b) the longitudinal diameter from the abdominal wall to the backside pelvic wall, which corresponded to the location of the pelvic inlet diameter, on a CT image (Fig. 1B). In this study, we defined pelvic width as the product obtained by multiplying (a) by (b). In order to obtain reproducibility, the measurement was repeated twice. Because pelvic width is not clinically difficult to measure, it is not necessary to perform additional examinations such as 3-dimensional CT.

Statistical Analysis

The Pearson χ^2 test with the level of significance set at 0.05 was performed to analyze the association among the risk factors and response variables, that is, operation time, intraoperative blood loss, creation of a covering ileostomy, and overall postoperative complications. A multiple logistic regression analysis was carried out to determine the risk factors associated with the response variables. As a model selection procedure in the regression analysis, a backward elimination procedure was used. All statistical analyses were performed with the Statistical Package for the Social Science (SPSS) version 13.0 for Windows software program (SPSS Inc., Chicago, IL).

RESULTS

Patient Clinicopathologic Characteristics

Sixteen of the 82 (19.5%) patients had lower rectal cancer (Rb), and the remaining 66 (80.5%) patients had upper or midrectal cancer (Table 1). Tumor invasion was up to the muscular layer (T1/2) in 29 (35.4%) of the patients, and the tumors in the remaining 53 (64.6%) were classified as T3/4. Preoperative radiotherapy (total 45 Gy) was performed in 5 (6.0%) of the 82 patients.

Perioperative Outcomes

The median operation time and the intraoperative blood loss were 300 minutes and 72.5 g, respectively. A covering ileostomy was created in 6 of the patients. Most patients without complications were discharged by postoperative day 14 or 18. Open conversion was required intraoperatively in 1 patient whose body mass index (BMI) was 33 kg/m^2 because of a poor recognition of intrapelvic space. Overall morbidity and mortality rates were 11.0% and 0%, respectively. Complications included wound infection (6.1%, n = 5), anastomotic leakage (1.2%, n = 1),

TABLE 2. Univariate Analysis for Predictive Factors Associated With an Operative Time of >300 Minutes

Variables	n	n (%)	*P*
Tumor size (mm)			0.24
≤40	43	21 (48.8)	
>40	39	24 (61.5)	
Sex			0.15
Male	49	30 (61.2)	
Female	33	15 (45.5)	
BMI (kg/m^2)			0.14
≤25	65	35 (53.8)	
>25	17	10 (58.8)	
VFA (cm^2)			0.98
≤100	42	23 (54.8)	
>100	40	22 (55)	
Dissection			0.25
D1/2	28	17 (60.7)	
D3	54	28 (51.9)	
Tumor location			0.57
Rs/Ra	64	35 (54.7)	
Rb	16	10 (62.5)	
Pelvic width (mm)			0.5
≤190	41	21 (51.2)	
>190	41	24 (58.5)	
T factor			0.023
T1/2	29	11 (37.9)	
T3/4	53	34 (64.2)	
Creating ileostomy			
Present	6	4 (66.7)	0.54
Absent	76	41 (53.9)	
Preoperative radiotherapy			0.24
Present	5	4 (80)	
Absent	77	41 (53.2)	
Comorbidity			0.84
Present	32	18 (56.3)	
Absent	50	27 (54)	

Multivariate analysis for predictable factors associated with an operative time of >300 min

	Variables	*P*	Regression Coefficient	SE	OR	95% CI
Operation time (>300 min)	T factor	0.011	1.279	0.503	3.59	1.34-9.64

BMI indicates body mass index; CI, confidence interval; OR, odds ratio; VFA, visceral fat index.

ileus (1.2%, n = 1), and pneumonia (2.4%, n = 2). Complications of wound infection, ileus, and pneumonia were managed conservatively. One patient with leakage required additional surgery.

Risk Factors Associated With an Operation Time >300 Minutes

Univariate analyses showed that only T factor affected an operation time of >300 minutes following Lap-AR (Table 2). Furthermore, a multivariate analysis identified T factor (odds ratio = 3.59, 1.34 < 95% confidence interval < 9.64, $P = 0.011$) as the independent risk factor associated with an operation time of >300 minutes.

Risk Factors Associated With an Intraoperative Blood Loss of >100 g

Univariate analyses showed that male sex, BMI > 25 kg/m^2, and VFA > 100 cm^2 affected an intraoperative blood loss of >100 g following Lap-AR (Table 3). Furthermore, multivariate analysis identified BMI (odds ratio = 3.75, 1.22 < 95% confidence interval < 11.54, $P = 0.012$) as the independent risk factor associated with an intraoperative blood loss of >100 g.

Factors Associated With the Creation of a Covering Ileostomy

Univariate analyses showed that a tumor size of >40 mm, Rb tumor location, and preoperative radiotherapy affected the creation of a covering ileostomy to prevent anastomotic leakage (Table 4). However, no independent factors associated with the creation of a covering ileostomy could be identified because of an insufficient number of patients (n = 6) for multivariate analysis.

Risk Factors Associated With Overall Postoperative Complications

Univariate analyses showed that a VFA of >100 cm^2, preoperative radiotherapy, and creating ileostomy affected overall postoperative complications following Lap-AR (Table 5). However, no independent risk factors associated with overall complications could be identified because of an insufficient number of patients (n = 9) for multivariate analysis.

DISCUSSION

Laparoscopic approaches are accepted for colon cancer surgery. Several randomized clinical trials (RCTs) of laparoscopic versus open colectomy for colon or colorectal

TABLE 3. Univariate Analysis for Predictive Factors Associated With an Operative Blood Loss of >100 g

Variables	n	n (%)	P
Tumor size (mm)			0.42
≤40	43	15 (34.9)	
>40	39	17 (43.6)	
Sex			0.024
Male	49	24 (49)	
Female	33	8 (24.2)	
BMI (kg/m^2)			0.015
≤25	65	21 (32.3)	
>25	17	11 (64.7)	
VFA (cm^2)			0.014
≤100	42	11 (26.2)	
>100	40	21 (52.5)	
Dissection			0.14
D1/2	28	14 (50)	
D3	54	18 (33.3)	
Tumor location			0.36
Rs/Ra	64	24 (37.5)	
Rb	16	8 (50)	
Pelvic width (mm)			0.65
≤190	41	15 (36.6)	
>190	41	17 (41.5)	
T factor			0.61
T1/2	29	10 (34.5)	
T3/4	53	22 (41.5)	
Creating ileostomy			0.15
Present	6	4 (66.7)	
Absent	76	28 (36.8)	
Preoperative radiotherapy			0.053
Present	5	4 (80)	
Absent	77	28 (36.4)	
Comorbidity			0.24
Present	32	15 (46.9)	
Absent	50	17 (34)	

Multivariate Analysis for Predictable Factors Associated With an Operative Blood Loss of >100 g

	Variables	P	Regression Coefficient	SE	OR	95% CI
Operative blood loss (>100 g)	BMI (>25)	0.021	1.323	0.573	3.75	1.22-11.54

BMI indicates body mass index; CI, confidence interval; OR, odds ratio; VFA, visceral fat index.

TABLE 4. Univariate Analysis for Predictive Factors Associated With the Creation of a Covering Ileostomy

Variables	n	n (%)	P
Tumor size (mm)			0.015
≤40	43	6	
>40	39	0	
Sex			0.72
Male	49	4	
Female	33	2	
BMI (kg/m^2)			0.8
≤25	65	5	
>25	17	1	
VFA (cm^2)			0.43
≤100	42	4	
>100	40	2	
Dissection			0.35
D1/2	28	1	
D3	54	5	
Tumor location			<0.001
Rs/Ra	66	0	
Rb	16	6	
Pelvic width (mm)			0.09
≤190	41	5	
>190	41	1	
T factor			0.91
T1/2	29	2	
T3/4	53	4	
Preoperative radiotherapy			<0.001
Present	5	4	
Absent	77	2	
Comorbidity			0.24
Present	32	1	
Absent	50	5	

BMI indicates body mass index; VFA, visceral fat index.

cancer suggest equivalent long-term outcomes with both techniques. However, the majority of these trials were conducted in patients with colon rather than rectal cancer.[1–7] In experienced hands, the goals of adequate oncologic surgery by laparoscopic methods (eg, lymph node removal, negative resection margins) can be met, and short-term outcomes appear comparable to those of open surgery.[18–21] Critical complications requiring reoperation and interventional treatment after minimally invasive surgery often distress the patient, the patient's family, and the surgeon. Therefore, we set out to evaluate the risk factors associated with short-term clinical outcomes of Lap-AR for rectal cancer.

In the present study, the depth of tumor invasion was found to be an independent risk factor associated with increased operative time, which might be related to the necessity of D3 lymph node dissection and the tumor size. It is common in pelvic surgery to consider a narrow pelvis to be 1 important factor associated with increased operative time. Targarona et al[22] reported that a smaller pelvic diameter was an independent predictor of operative time in a series of 60 patients who underwent laparoscopic resection for rectal tumors, and Akiyoshi et al[23] also reported a narrow pelvis to be an independent predictor of operative time in 79 patients undergoing laparoscopic resection for lower rectal cancer. However, the present study did not show an association between the pelvic width and an increased operative time. Although we usually perform pelvic surgery without preoperative pelvimetry, in the near future, an easy method to measure pelvic volume may be necessary to perform laparoscopic surgery more safely.

Surgeons have known for some time that laparoscopic colorectal surgery is clearly more technically demanding in obese patients.[24] In the present study, BMI was identified as an independent risk factor for operative blood loss, whereas VFA was not found to be an important risk factor associated with an increase in operative blood loss. Some reports indicate that BMI is the better predictor of surgical outcome,[24,25] whereas other reports indicate that VFA is a better predictor than BMI.[26,27] To decrease operative blood loss, it is necessary to use ultrasonic shears and bipolar devices. We use the Sonosurg (Olympus, Tokyo, Japan) and LigaSure systems (Covidien, Neustadt an der Weinstrasse, Germany) for dissecting several tissues except for major vessels. Use of such devices may contribute to the spread of laparoscopic surgery in obese patients. Further examination is necessary to determine with certainty as to which is the better predictor of surgical outcome.

In the present study, univariate analysis showed that a VFA of >100 cm^2 and preoperative radiotherapy affected overall postoperative complications after Lap-AR. However, no independent risk factors associated with overall complications could be identified because of an insufficient sample size for multivariate analysis. Akiyoshi et al[28] reported that Lap-AR after preoperative chemoradiation therapy does not appear to affect surgical and oncologic outcomes negatively.

The capability to create a covering ileostomy during AR is the 1 thing that most differentiates AR from gastrectomy.

TABLE 5. Univariate Analysis for Predictive Factors Associated With Overall Postoperative Complications

Variables	n	n (%)	P
Tumor size (mm)			0.22
≤40	43	3 (7)	
>40	39	6 (15)	
Sex			0.058
Male	49	8 (16)	
Female	33	1 (3)	
BMI (kg/m^2)			0.32
≤25	65	6 (9.2)	
>25	17	3 (18)	
VFA (cm^2)			0.012
≤100	42	1 (2.3)	
>100	40	8 (20)	
Dissection			0.96
D1/2	28	3 (10.7)	
D3	54	6 (11.1)	
Tumor location			0.83
Rs/Ra	66	7 (10.6)	
Rb	16	2 (12.5)	
Pelvic width (mm)			0.72
≤190	41	4 (8.9)	
>190	41	5 (12.2)	
T factor			0.38
T1/2	29	2 (6.8)	
T3/4	53	7 (13.2)	
Creating ileostomy			0.002
Present	6	3 (50)	
Absent	76	6 (7.9)	
Preoperative radiotherapy			0.032
Present	5	2 (40)	
Absent	77	7 (9.1)	
Comorbidity			0.069
Present	32	1 (3.1)	
Absent	50	8 (16)	

BMI indicates body mass index; VFA, visceral fat index.

During AR, surgeons can create a temporary covering ileostomy to protect the colorectal anastomosis. It is difficult for surgeons to decide on when to create an ileostomy. The 3 experienced surgeons in the present study created a covering ileostomy in 6 of 82 (7.3%) patients. Univariate analysis showed that > 40-mm tumor size, Rb tumor location, and preoperative radiotherapy affected the creation of a covering ileostomy. The reason that tumor size was a significant factor in univariate analysis may be due to the decrease in tumor size as a result of preoperative radiotherapy, whereas the anal margin of the tumor in all patients receiving covering ileostomy progressed to the anal canal. Unfortunately, an insufficient sample size for multivariate analysis prevented the identification of independent risk factors associated with the creation of a covering ileostomy.

Although Law et al[19] reported on a prospective RCT comparing open and Lap-AR for upper rectal cancerand Akiyoshi et al[23] reported factors affecting the difficulty of laparoscopic surgery for low rectal cancer, unfortunately, their patients were confined to upper or lower rectal cancer only. In the present study, we evaluated the short-term clinical outcomes of Lap-AR for all rectal cancer locations, whereas middle and lower rectal cancer were excluded in most large-scale RCTs. Lujan et al[20] reported an RCT comparing laparoscopic and open surgery in patients with rectal cancer. In their study, however, a covering ileostomy was created in more than 60% of the patients, and abdominoperineal excisions were performed in 23.8% of the patients in the laparoscopic group. Our aim was the evaluation of predictive factors for short-term clinical outcomes of Lap-AR that required anastomosis. To our knowledge, there are few reports that discuss the predictors of surgical outcomes of Lap-AR comprehensively. We recognized the limitations of a retrospective study. Nevertheless, we believe that this series provides important information for beginners in selecting a suitable case in the initial learning curve of the laparoscopic treatment of rectal cancer. Further work to refine the predictive factors of surgical outcomes and validate these factors with other data sets should be undertaken.

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Visinin-like protein-1 overexpression is an indicator of lymph node metastasis and poor prognosis in colorectal cancer patients

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Lymph node metastasis is an important factor determining outcome from colorectal cancer (CRC). Identification of molecular markers useful to predict lymph node metastasis is urgently needed. Our objective was to identify genes useful for characterization and prediction of lymph node metastasis in CRC. Gene expression profiles of cancer were determined by human U133 Plus 2.0 GeneChip® in 24 CRC patients, and patients with and without lymph node metastasis were compared. We focused on the visinin-like protein-1 (VSNL-1) gene and evaluated VSNL-1 mRNA expression levels with reverse transcriptase-polymerase chain reaction and immunohistochemical methods. Immunohistochemical evaluation of VSNL-1 mRNA expression was also performed in 143 other CRC patients to determine clinicopathological significance of VSNL-1. Twenty-four novel discriminating genes showed expression significantly different between patients with and without lymph node metastasis. Mean level of VSNL-1 mRNA expression in tumor tissue with lymph node metastasis was significantly higher than that in tissue without lymph node metastasis. Immunohistochemical examination demonstrated immunoreactivity for VSNL-1 in cytoplasm of the cancer cells with lymph node metastasis. High VSNL-1 expression was significantly associated with lymphatic invasion in stage II disease ($p = 0.0061$) and number of lymph node metastases in stage III disease ($p = 0.0461$). Patients with high VSNL-1 expression had significantly poorer prognosis than those with low expression in stage III disease ($p = 0.045$). This study is the first to demonstrate a prognostic role for VSNL-1 at the mRNA level, suggesting the possible usefulness of VSNL-1 as a predictor of lymph node metastasis and poor prognosis in CRC.

The ability of malignant tumors to metastasize is the major cause of death from cancer and failure of cancer treatment. Although treatments with radical surgery, radiotherapy and systemic chemotherapy are performed, clinical outcome is unsatisfactory because of metastatic spread. It has been shown that tumor metastatic stage is one of the most important prognostic factors of clinical outcome.[1] Understanding the molecular mechanisms of the complex multistep process of tumor metastasis can facilitate the development of preventive measures, early diagnostic methods and better treatment. The morbidity and mortality rates of colorectal cancer (CRC) in many Asian countries, including Japan, have gradually increased every year.[2] Chemotherapy has improved the clinical outcome and survival rate in cases of nonresectable and recurrent CRC;[3,4] however, there is still a need to identify patient subgroups with high or low risk of tumor recurrence and to tailor individual therapeutic interventions. In particular, lymph node metastasis is widely accepted as one of the most important prognostic factors in CRC patients,[5] and there is an urgent need to identify molecular markers that can be used as predictors of lymph node metastasis.

Presently, the standard procedure for determining the spread of metastatic disease to lymph nodes is pathological examination of ~20 resected lymph nodes stained with hematoxylin and eosin (H&E). Generally, each lymph node is transected at the maximum cut and examined by H&E staining. However, false negatives occur at a constant rate of probability, even though examination for micrometastasis, which is defined as the presence of tumor cells—single cells or in small clusters—and is detected only by cytokeratin-specific immunostaining, is performed. We reasoned that an assay that is able to identify patients with metastatic disease by measuring RNA expression levels of selected genes would be helpful in clinical decision making. There are several reports of potential markers associated with lymph node metastasis;[6–9] however, none of these markers is yet ready for clinical application. According to our study of microarray

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Table 1. Clinicopathological features of patients positive and negative for lymph node metastasis by microarray analysis

	pN+ ($n = 13$)		pN− ($n = 11$)	
Factors	**No.**	**%**	**No.**	**%**
Age (mean, range)	67.3 (55–82)		67.1 (45–81)	
Sex (male:female)	5/8		7/4	
Histological grade				
Well	7	53.8	4	36.4
Moderately, poorly, others	6	56.2	7	63.6
Size				
≤50 mm	7	53.8	5	45.5
>51 mm	6	56.2	6	54.5
Tumor invasion				
sm, mp, ss, se, A	11	84.6	11	100.0
si, Ai	2	15.4	0	0.0
Number of lymph node metastases				
N0	–		11	100.0
N1 (1–3)	8	61.5	–	
N2 (≥4)	5	38.5	–	
Lymphatic invasion				
Absent	1	7.7	9	81.8
Present	12	92.3	2	18.2
Venous invasion				
Absent	3	23.0	6	54.5
Present	10	77.0	5	45.5
Stage				
I	0	0.0	6	54.5
II	0	0.0	5	45.5
III	13	100.0	0	0.0

Tumor invasion of submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se) and invasion of adjacent structures (si). A: Tumor with serosal invasion in rectum below the peritoneal reflexion. Ai: Tumor with invasion into adjacent organs in rectum below the peritoneal reflexion.

and reverse transcriptase-polymerase chain reaction (RT-PCR) data, we identified two important genes associated with lymph node metastasis. One was VSNL-1 that had been described as a tumor suppressor gene in esophageal squamous cell carcinoma by Mahloogi *et al.*[10] or as an oncogene in neuroblastoma by Xie *et al.*,[11] whereas the other was ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) that had been previously reported to play roles in mineralization, nucleotide recycling and insulin resistance.[12] The immunohistochemical examinations demonstrated that not ENPP-1 but VSNL-1 had good correlations between mRNA expression and protein expression, which meant significant correlations between protein expression and pathological lymph node metastasis. Therefore, in our study, we focused on the clinicopathological significance of the expression of visinin-like protein-1 (VSNL-1) in CRC and evaluated the prognostic implications of VSNL-1 expression in stage II and stage III disease.

Material and Methods

Clinical samples

Medical records were searched for patients who underwent surgical resection for CRC at Oita University between 2009 and 2010. Tissue from 24 CRC patients with information about clinicopathological features was collected. Of these 24 patients, 13 had pathological lymph node metastasis and 11 did not. Clinical characteristics of the patients are summarized in Table 1. Snap-frozen primary tumor and paired non-tumor tissue specimens were immediately cut from resected colon and rectum and embedded in Tissue Tek OCT medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen and kept at −80°C until RNA extraction. Analyzed samples were taken from the primary tumor, not from a lymph node. Formalin-fixed paraffin-embedded samples from 143 consecutive CRC patients who underwent colorectal surgery at Oita University between 2001 and 2005 were also examined immunohistochemically to investigate the association between

clinicopathological features and VSNL-1 expression. Written informed consent was obtained from all patients, and the study protocol was approved by the local ethics committee.

Total RNA extraction and first-strand cDNA synthesis

First, frozen specimens were examined microscopically by H&E staining after sectioning with a Cryostat microtome, and we confirmed that cancer cells accounted for more than 95% of the cells within the specimen. Second, frozen tissue specimens were homogenized, and total RNA was extracted with a miRNeasy mini kit (Qiagen, Valencia, CA). Total RNA (8.0 μg) was reverse transcribed to cDNA using MLV reverse transcriptase (Invitrogen Corp., Carlsbad, CA).

cDNA microarray

We used the commercially available Human Whole Genomic Oligo DNA Microarray Kit (Agilent Technologies, Santa Clara, CA). Cyanine (Cy)-labeled cRNA was prepared using T7 linear amplification as described in the Agilent Low RNA Input Fluorescent Linear Amplification Kit Manual (Agilent Technologies). Labeled cRNA was fragmented and hybridized to an oligonucleotide microarray (Whole Human Genome 4x44 K; G4112F, Agilent Technologies). The fluorescent intensities were determined with an Agilent DNA Microarray Scanner and analyzed using G2567AA Feature Extraction Software Version A.7.5.1 (Agilent Technologies), which used the LOWESS (locally weighed linear regression curve fit) normalization method.[13] This microarray study followed the MIAME guidelines issued by the Microarray Gen Expression Data group.[14] Further analyses were performed using GeneSpring version 7.3 (Silicon Genetics, San Carlos, CA).

Quantitative RT-PCR

The primers used to generate cDNA are summarized in Supporting Information Table 1. Glyceraldehyde-3-phosphatedehydrogenase (GAPDH), the internal control, was amplified using 5′-GCTCTCTGCTCCTCCTGTTC-3′ (sense) and 5′-ACGACCAAATCCGTTGACTC-3′ (antisense). Real-time monitoring of PCR reactions was performed with a LightCycler System (Roche Applied Science, Indianapolis, IN) and SYBR-Green I dye (Roche Applied Science). After the reaction mixture was loaded into glass capillary tubes, quantitative RT-PCR was performed with the following cycling conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, annealing at 62°C for 10 sec and extension at 72°C for 10 sec. After amplification, the products were subjected to a temperature gradient of 67 to 95°C in increments of 0.2°C/sec under continuous fluorescence monitoring to produce a melting curve of the products.

Quantitative RT-PCR data analysis

We used LightCycler Software version 3.5 (Roche Molecular Biochemicals, Indianapolis, IN) to calculate the cycle numbers. After proportional baseline adjustment, the fit point method was used to determine the cycle in which the linear signal was first distinguishable from the baseline. This cycle number was used as the crossing point value. A standard curve was produced by measuring the crossing point of each standard value and plotting it against the logarithmic value concentration. Concentrations of unknown samples were calculated by plotting their crossing points against the standard curve and dividing by GAPDH content. GAPDH expression was not different between tumor and normal tissues.

Immunohistochemistry

Immunohistochemical studies for VSNL-1 and ENPP-1 were performed on formalin-fixed, paraffin-embedded surgical sections obtained from the 24 CRCs that had been analyzed by microarray and the 143 CRCs that had been resected surgically between 2001 and 2005. Tissue sections were deparaffinized, soaked in 0.01 M sodium citrate buffer and cell antigens were retrieved. The primary rabbit polyclonal antibodies against VSNL-1 (Roche Diagnostics Japan, Tokyo, Japan; cat. no. 04688589001) and the goat polyclonal antibodies against ENPP-1 (Abcam, Tokyo; cat. no. ab40003) were used at dilutions of 1:50. Tissue sections were immunohistochemically stained using the avidin-biotin-peroxidase method (LSAB + system-HRP; DAKO, Kyoto, Japan). All sections were counterstained with hematoxylin. Evaluation of the immunohistochemical data was performed by two independent pathologists who were blinded to the clinical and pathological data of the patients. Staining intensity was judged as 0 (no stain), 1 (less than that in normal colorectal epithelial cells as the positive internal control), 2 (as intense as that in the positive internal control) or 3 (more than that in the positive internal control). The populations of positively stained cells were categorized into four groups on the basis of the percentage of positive tumor cells: 0 (0% positive cells), 1 (1–9% positive cells), 2 (10–50% positive cells) and 3 (>50% positive cells). Scoring was performed three times per case in three distinct fields, and the three scores were averaged. The averaged scores for intensity and population were summed, and summed scores of >3 or ≤ 2 were categorized as high or low, respectively. Although there were a few differences as to each scores: staining intensity and populations of positively stained cells between the two pathologists, there were no differences as to the final judgment, which indicated high or low group in immunohistochemical staining examination between the two pathologists.

Association between VSNL-1 expression and clinicopathological features in CRCs

The association between clinicopathological features and VSNL-1 expression in the 143 consecutive CRC patients who underwent colorectal surgery at Oita University between 2001 and 2005 was examined. Clinicopathological features included histological grade, tumor size, tumor invasion, the number of lymph nodes examined and lymphatic invasion.

Table 2. Representative mRNAs differentially expressed in tumor cells with lymph node metastasis

ID (mRNABASE 9.0)	Fold change	*p*-value	Gen symbol	GenBank accession
(A) Downregulated mRNA in tumor cells with lymph node metastasis				
A_23_P77043	3.33	0.0439	CATSPERB	NM_024764
A_23_P17438	3.22	0.0293	EDN3	NM_207032
A_23_P200670	2.48	0.0392	WDR78	NM_207014
A_24_P595460	2.58	0.0082	NUCB2	AK097398
A_23_P89798	2.07	0.0025	FECH	NM_001012515
A_23_P259442	3.39	0.047	CPE	NM_001873
A_23_P12733	2.15	0.0255	H2AFY2	NM_018649
A_24_P116587	2.38	0.0093	SEZ6L2	NM_201575
A_32_P16315	2.62	0.0499	RP11-631M21.2	NM_001164154
A_23_P26854	2.60	0.0411	RICH2	NM_014859
A_24_P88801	2.73	0.0113	NPHP1	NM_000272
A_32_P192376	3.70	0.0332	ENPP1	NM_006208
A_24_P211106	2.64	0.0241	TNFRSF11A	AB209762
(B) Upregulated mRNA in tumor cells with lymph node metastasis				
A_23_P209978	3.71	0.0375	VSNL1	NM_003385
A_24_P226322	2.03	0.0366	SH3BGRL2	NM_031469
A_23_P348737	2.94	0.0224	NR2F1	NM_005654
A_23_P3552	2.13	0.0314	RRN3P1	BC068999
A_23_P132378	2.24	0.0421	CELSR1	NM_014246
A_23_P116173	3.50	0.0344	C11orf93	NM_001136105
A_23_P384044	2.17	0.0223	CNIH3	NM_152495
A_23_P118122	2.51	0.0332	RGS11	NM_003834
A_32_P405703	2.07	0.0223	KIAA1161	NM_020702
A_24_P921446	2.11	0.0113	EMP1	BC017854
A_23_P45324	3.44	0.0449	TMEM35	NM_021637

Additionally, we evaluated the prognostic implications of VSNL-1 expression in stage II and stage III disease.

Statistical analysis

For continuous variables, data are expressed as the means + standard deviation. The relation between VSNL-1 mRNA expression and clinicopathological factors was analyzed using a χ^2 test and *t*-test. Overall survival curves were plotted according to the Kaplan–Meier method and measured from the day of surgery, with the log-rank test applied for comparison. Variables with p value < 0.05 by univariate analysis were used in subsequent multivariate analyses based on Cox's proportional hazards model. All statistical analyses were performed with JMP software (SAS Institute, Cary, NC), and the findings were considered significant when the p value was <0.05.

Results

Identification of genes differentially expressed in primary CRCs with lymph node metastasis

First, to identify the genes differentially expressed in primary CRCs with lymph node metastasis compared with those without metastasis or normal colorectal epithelium, we performed gene expression profiling on the 24 cases of CRC, which consisted of 13 pathological node-positive cases and 11 node-negative cases. Additionally, eight normal colorectal epithelial samples were collected from four of the pathological node-positive patients and four of the node-negative patients and were analyzed.

Comparison of the expression levels of genes as described in the Material and Methods section revealed that 24 genes were differentially expressed (Table 2), of which 13 genes were significantly downregulated in the patients with lymph node metastasis, and 11 genes were upregulated.

Quantitative RT-PCR

To validate the microarray data, the 24 genes were further analyzed by quantitative RT-PCR. The expression level of all 24 genes showed similar trends in microarray and quantitative RT-PCR results (Supporting Information Fig. 1). Among them, VSNL-1 and ENPP1 showed statistically significant differences in their expressions between CRCs with lymph node metastasis and those without metastasis (Fig. 1).

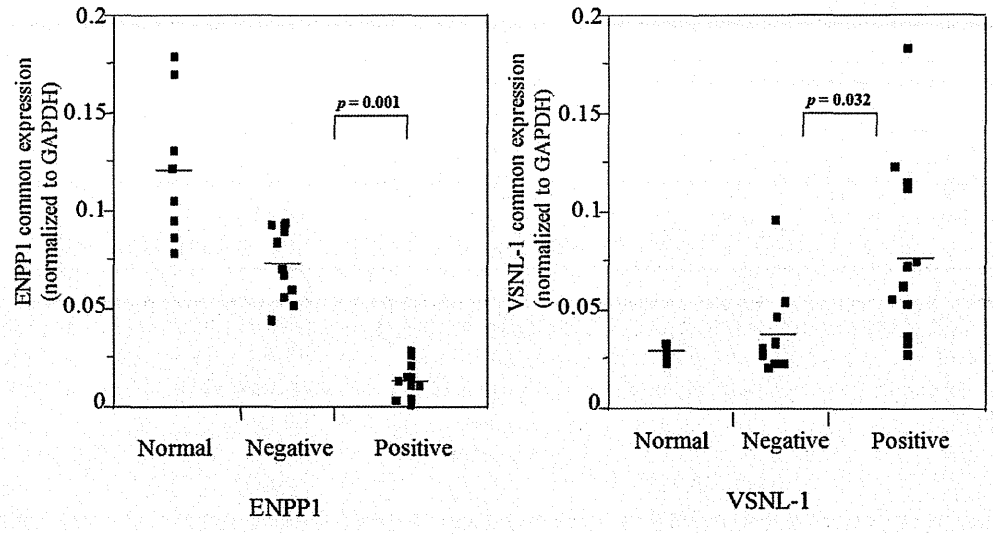

Figure 1. Among 24 genes, quantitative RT-PCR data of visinin-like protein 1 (VSNL-1, right panel) and ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1, left panel) exhibited statistically significant differences of their expression between colorectal cancers with lymph node metastasis and those without metastasis.

Immunohistochemical examination

To determine the expression levels of VSNL-1 and ENPP1 proteins in CRC tissues, we performed immunohistochemical analysis using specific antibodies against each protein. First, VSNL-1 protein was expressed at only low levels in the normal colorectal epithelium adjacent to the tumor in all 24 CRCs analyzed (Fig. 2*a*). In contrast, tumor cells in 11 (45.8%) of the 24 CRCs exhibited high VSNL-1 immunoreactivity in the cytoplasm, as indicated by high immunostaining with the anti-VSNL-1 antibody. Among these 11 CRCs (Fig. 2*b*), nine had been diagnosed pathologically as lymph-node positive and the other two as lymph-node negative. Furthermore, among the 13 CRCs whose tumor cells showed low to negative expression of VSNL-1 (Fig. 2*c*), only four had been diagnosed as lymph-node positive. These findings demonstrated that VSNL-1 had good correlations between mRNA expression and protein expression, which meant there were significant correlations between protein expression and pathological lymph node metastasis (Fig. 2*d*, Supporting Information Table 2). On the other hand, ENPP-1 did not have good correlation (data not shown). Therefore, we focused on the clinicopathological significance of VSNL-1 expression in CRC.

Relation between VSNL-1 expression and clinicopathological features in CRCs. To determine whether the expression of VSNL-1 protein correlates with clinicopathological status other than lymph node metastasis, we immunohistochemically examined the expression of VSNL-1 in the additional 143 cases of CRC. On the basis of VSNL-1 expression levels, the 143 cases were divided into two groups: the high VSNL-1 expression group ($n = 74$) and the low VSNL-1 expression group ($n = 69$). The association between VSNL-1 expression and clinicopathological features is summarized in Table 3. The levels of VSNL-1 expression, the frequency of lymphatic vessel invasion and vascular invasion and stage classification as well as lymph node metastasis were much more elevated in the high VSNL-1 expression group than in the low VSNL-1 expression group ($p < 0.05$).

To investigate the clinicopathological significance of VSNL-1 for stage II CRC, we evaluated the association between VSNL-1 expression and clinicopathological features. The low VSNL-1 expression group comprised 51 patients, and the high VSNL-1 expression group comprised 22 patients (Table 4). Although the expression of VSNL-1 correlated significantly with lymphatic invasion ($p = 0.0061$), there was no significant difference between expression of VSNL-1 and overall survival in stage II patients ($p = 0.308$; Fig. 3, left panel).

In our analysis of stage III disease, the low VSNL-1 expression group comprised nine patients, and the high VSNL-1 expression group comprised 47 patients (Table 4). Here, expression of VSNL-1 correlated significantly with both the number of lymph node metastases ($p = 0.0461$) and overall survival in stage III ($p = 0.045$; Fig. 3, right panel). Univariate analysis identified that VSNL-1 expression, tumor invasion, lymph node metastasis, lymphatic invasion and venous invasion were prognostic factors for overall survival after surgery. Variables with p value < 0.05 by univariate analysis were selected for multivariate analysis using Cox's proportional hazards model. VSNL-1 expression (relative risk: 2.38, confidence interval: 1.54–3.36, $p = 0.003$) was found to be a significant factor affecting overall survival after surgery (Table 4).

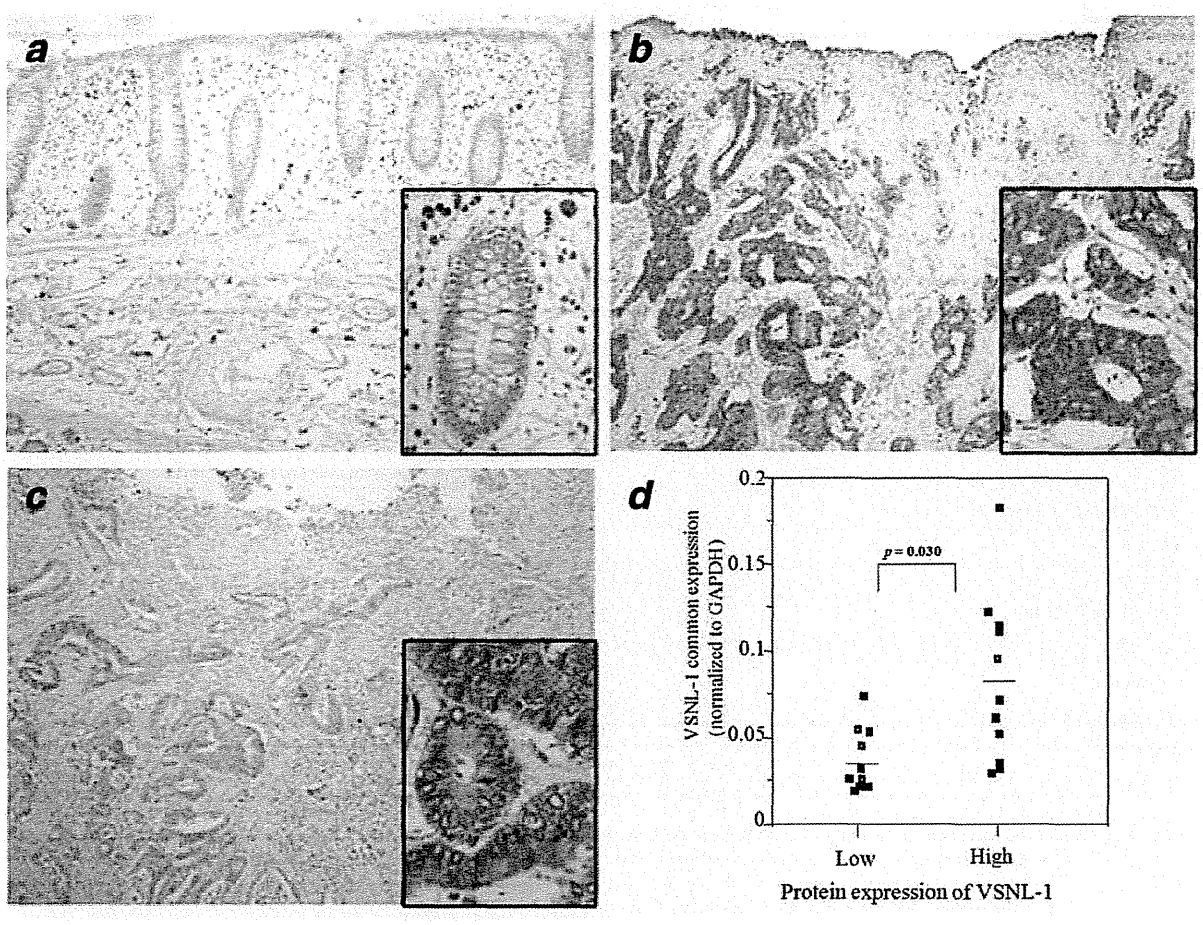

Figure 2. Immunohistochemistry for VSNL-1 in the normal colon and cancer cells. (*a*) Low to negative immunoreactivity is observed in the cytoplasm of non-neoplastic epithelial cells, original magnification ×20 and ×40. (*b*) High type: immunoreactivity is strongly detected in the cytoplasm of the cancer cells. (*c*) Low to negative immunoreactivity is observed in the cytoplasm of the cancer cells. (*d*) Relation between mRNA and protein expression in VSNL-1. VSNL-1 had good correlations between mRNA expression and protein expression.

Discussion

VSNL-1 is an EF-hand calcium-binding protein belonging to the neural Ca^{2+}-sensor protein family mainly expressed in the central nervous system. VSNL-1 is associated with membranes under physiological calcium concentrations.[15,16] A well-known effect of VSNL-1 in brain cells is to modulate the intracellular levels of cAMP, both the basic and extracellularly induced cAMP concentrations. In olfactory membranes, after odor stimulation, VSNL-1 preparations inhibited adenylate cyclase activity in a calcium-dependent manner.[17] Xie *et al.* reported that upregulation of VSNL-1 potentiated the anoikis-resistant ability of tumor cells in neuroblastoma, and the expression of anoikis inhibitor TrkB, intracellular adhesion molecule 1, major histocompatibility complex class I and CD44 and CD44v6 was associated with VSNL-1 level.[11] These results suggested that VSNL-1 has an important role in proliferative and invasive phenotypes, which contribute to CRC progression. However, Mahloogi *et al.* found that VSNL-1 expression in esophageal squamous carcinoma was reversely correlated with tumor invasive features, which may be due to VSNL-1 elevating the level of intracellular cyclic adenosine 3′,5′-monophosphate.[10] The elevation of cyclic adenosine 3′,5′-monophosphate plays both a positive[18] and negative role[19] in cell migration. The precise functions of VSNL-1 have not been clarified, and this protein probably plays different roles in squamous carcinoma and CRC. Our study of CRC suggested that VSNL-1 expression might have a positive effect on tumor growth and cell invasiveness as well as on neuroblastoma progression. Although VSNL-1 was associated with lymphovascular invasion in addition to lymph node metastasis and Dukes' classification, it was not associated with tumor size. We speculated that this was because VSNL-1 might play a role in invasion but not in tumor enlargement and expansion.

To date, there have been several reports of molecular markers associated with lymph node metastasis.[6–9] Wang *et al.*

Table 3. Clinicopathological significance of VSNL-1 expression in colorectal cancer

	Tumor low expression ($n = 69$)		Tumor high expression ($n = 74$)		
Factors	**No.**	**%**	**No.**	**%**	***p*-value**
Age (mean, range)	66.7 (24–94)		67.0 (42–93)		0.64
Sex (male:female)	42/27		38/36		0.252
Histological grade					0.582
Well	33	47.8	32	43.2	
Moderately, poorly, others	36	52.2	42	56.8	
Size					0.518
≤50 mm	41	59.4	40	54.1	
>51 mm	28	48.6	34	55.9	
Tumor invasion					0.158
sm, mp, ss, se, A	49	71.0	60	81.1	
si, Ai	20	29.0	14	28.9	
Lymph node metastasis					<0.0001
Absent	60	87.0	23	31.1	
Present	9	13.0	51	68.9	
Lymphatic invasion					<0.0001
Absent	42	60.9	18	24.3	
Present	27	39.1	56	75.7	
Venous invasion					0.0436
Absent	46	66.7	37	50.0	
Present	23	33.3	37	50.0	
Stage					<0.0001
I	9	13.0	0	0.0	
II	51	73.9	22	29.7	
III	9	13.1	47	63.5	
IV	0	0.0	5	6.8	

Tumor invasion of submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se) and invasion of adjacent structures (si). A: Tumor with serosal invasion in rectum below the peritoneal reflexion. Ai: Tumor with invasion into adjacent organs in rectum below the peritoneal reflexion.
Abbreviation: VSNL-1: visinin-like protein-1.

reported that underexpression of SATB2 correlated with lymph node metastasis.[6] On the other hand, three reports indicated that overexpression of each of these biomarkers, stanniocalcin 2,[7] IMP3[8] and FANCD2,[9] correlated with lymph node metastasis. However, these biomarkers have not reached the point of clinical application. There is a possibility for the clinical application of VSNL-1 because of its strong correlation with lymph node metastasis and poor prognosis. In our study, an analysis of overall survival curves in patients with stage III disease showed that patients in the high VSNL-1 expression group had a significantly poorer prognosis than those in the low expression group. Additionally, although there was no significant difference between expression of VSNL-1 and overall survival in stage II disease ($p = 0.308$), expression of VSNL-1 correlated significantly with lymphatic invasion ($p = 0.0061$). The reason for no significant difference in overall survival might have been due to an insufficient number of stage II patients.

The American Joint Committee on Cancer TNM staging system is currently the standard for determining the prognosis of patients with CRC. The role of systemic adjuvant chemotherapy in CRC patients with lymph node metastasis has been established in a large number of clinical trials. However, its role in CRC patients at stage II has not been well established yet.[20–22] A recent meta-analysis of prospective randomized clinical trials of adjuvant chemotherapy in patients with Dukes' B tumors, which do not have lymph node metastasis, has not shown a survival benefit of chemotherapy,[23] but a significant proportion of Dukes' B patients (25–30%) develop recurrence and metastasis, resulting in cancer death.[24] In contrast, adjuvant treatment is universally recommended for all patients with stage III CRC.[25] However,

Table 4. Clinicopathological significance of VSNL-1expression in stage II and III disease

	Stage II				
	Tumor low expression ($n = 51$)		Tumor high expression ($n = 22$)		
Factors	**No.**	**%**	**No.**	**%**	***p*-value**
Age (mean, range)	65.4 (24–94)		67.2 (42–88)		0.71
Sex (male:female)	31/20		11/11		0.392
Histological grade					0.70
Well	23	45.1	11	50.0	
Moderately, poorly, others	28	54.9	11	50.0	
Size					0.315
≤50 mm	30	58.8	15	68.1	
>51 mm	21	41.2	6	31.9	
Tumor invasion					0.742
ss, se, A	40	78.4	18	81.8	
si, Ai	11	21.6	4	18.2	
Number of lymph node examined (median, range)	18.5 (6–33)		17.0 (8–29)		0.288
Lymphatic invasion					0.0061
Absent	36	76.4	8	36.4	
Present	15	23.6	14	63.6	
Venous invasion					0.092
Absent	38	74.5	12	54.5	
Present	13	25.5	10	45.5	

	Stage III				
	Tumor low expression ($n = 9$)		Tumor high expression ($n = 47$)		
Factors	**No.**	**%**	**No.**	**%**	***p*-value**
Age (mean, range)	67.4 (57–81)		67.1 (45–93)		0.61
Sex (male:female)	6/3		25/22		0.456
Histological grade					0.115
Well	6	33.3	18		
Moderately, poorly, others	3	66.7	29		
Size					0.716
≤50 mm	4	44.4	24	51.1	
>51 mm	5	55.6	23	48.9	
Tumor invasion					0.186
sm, mp, ss, se, A	6	33.3	40	15.0	
si, Ai	3	66.7	7	85.0	
Number of lymph node examined (median, range)	19.0 (10–32)		19.5 (11–28)		0.441
Number of lymph node metastases					0.0461
N1 (1–3)	8	88.9	25	53.2	
N2 (≥4)	1	11.1	22	46.8	
Lymphatic invasion					0.066
Absent	4	44.4	8	17.0	
Present	5	45.6	39	83.0	

Table 4. Clinicopathological significance of VSNL-1expression in stage II and III disease (Continued)

	Stage III				
	Tumor low expression ($n = 9$)		Tumor high expression ($n = 47$)		
Factors	**No.**	**%**	**No.**	**%**	***p*-value**
Venous invasion					0.916
Absent	4	44.4	20	42.6	
Present	5	45.6	27	47.4	
Postoperative chemotherapy					0.436
Performed	9	100.0	44	93.6	
Not performed	0	0.0	3	6.4	

Univariate and multivariate analysis of clinicopathological factors for overall survival in stage II and III (Cox proportional regression model)

	Univariate analysis			Multivariate analysis		
Factors	**PR**	**95% CI**	***p*-value**	**PR**	**95% CI**	***p*-value**
Age (≤65/>65)	0.98	0.55–1.41	0.55	–	–	–
Sex (male/female)	1.22	0.89–1.93	0.35	–	–	–
Histology	1.33	0.93–1.98	0.15	–	–	–
Tumor size	1.29	0.98–2.33	0.32	–	–	–
Depth of tumor invasion (sm, mp, ss, se, A/Si, Ai)	1.88	1.55–3.01	0.022	1.18	1.08–3.13	0.028
Lymph node metastasis (negative/positive)	2.72	1.88–4.02	0.001	2.63	1.41–3.64	0.001
Lymphatic invasion (negative/positive)	1.95	1.81–4.14	0.001	1.92	1.21–2.77	0.021
Venous invasion (negative/positive)	1.54	1.22–2.44	0.028	1.77	1.10–2.73	0.032
VSNL-1 expression (low/high)	2.21	1.53–2.39	0.032	2.38	1.54–3.36	0.003

Tumor invasion of submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se) and invasion of adjacent structures (si). A: Tumor with serosal invasion in rectum below the peritoneal reflexion. Ai: Tumor with invasion into adjacent organs in rectum below the peritoneal reflexion. Abbreviation: VSNL-1: visinin-like protein-1.

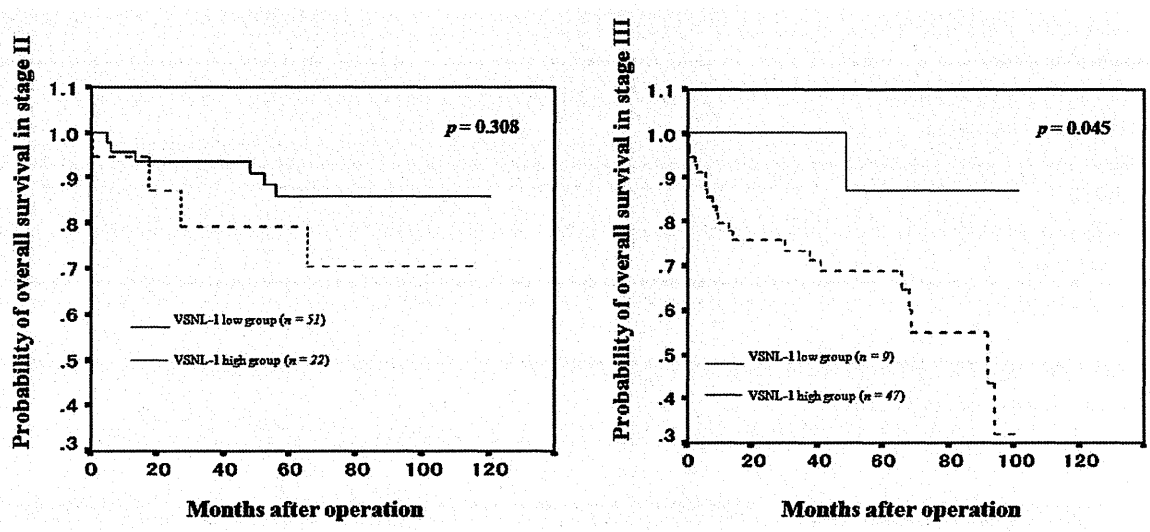

Figure 3. Overall survival curves for colorectal cancer patients based on the level of VSNL-1 expression in stage II and III disease. There were no significant differences in survival rates between the low expression group and the high expression group ($p = 0.308$, log-rank test) in stage II disease (left panel). Low expression group ($n = 51$) and high expression group ($n = 22$). However, the overall survival rate for patients in the low expression group was higher than that for patients in the high expression group ($p = 0.045$, log-rank test) in stage III disease (right panel). Low expression group ($n = 9$) and high expression group ($n = 47$).

patients with T1-T2N1M0 (stage IIIA) tumors have significantly higher survival rates than do patients with stage IIIB tumors,[26] suggesting that selection of adjuvant chemotherapy requires optimization. Therefore, there exists a need to identify patients who would benefit from adjuvant chemotherapy before treatment, and molecular markers may give us important insight into this challenge[27,28] as the patients' markers can be taken into account when selecting the appropriate chemotherapy. Presently, TNM-G staging (G means genetic diagnosis), a concept in which biological molecular markers may be helpful for making decisions in addition to TNM staging, has emerged. Because pathological examination of lymph node metastasis is limited even though examination of micrometastasis using immunohistochemical staining is performed, measuring RNA expression levels of select genes would be helpful. VSNL-1 might be a novel marker for prognosis of CRC, especially reflecting the potential for malignancy in stage II or stage III disease.

Recently, the development of novel therapies that target critical biological pathways has greatly expanded treatment options for patients with CRC and has shown substantial improvement in survival.[29] Antagonization of VSNL-1 might become a new molecular target agent. Moreover, because VSNL-1 is overexpressed only in CRC cells but is not expressed in normal cells, it may be a novel target for cancer treatment without harming normal colorectal cells. Of course, the clarification of functions of VSNL-1 in normal and cancer cells is necessary for it. This result provides hope regarding the development of novel target therapies without adverse effects.

In conclusion, using mRNA microarrays, we identified genes specifically expressed in CRC cells that correlated with lymph node metastasis. We chose VSNL-1 from the candidate genes and evaluated VSNL-1 expression in 143 CRC patients. In comparison with low VSNL-1 expression, high VSNL-1 expression was significantly associated with a high rate of lymph node metastasis and poor patient prognosis. The results of our study suggest that VSNL-1 might be a predictor of lymph node metastasis and poor prognosis. Although further studies of VSLN-1 in animal models will be needed to clarify its mechanisms, VSLN-1 has the possibility of becoming a novel molecular marker in CRC.

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