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Clinicopathological and Prognostic Significance of FOXM1 Expression in Esophageal Squamous Cell Carcinoma

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Abstract. *Background: Esophageal squamous cell carcinoma (ESCC) has a poor prognosis because invasion and metastasis are prevalent. To improve diagnosis, it is important to identify and characterize tumor-specific molecular markers in ESCC. FOXM1 is overexpressed and correlates with pathogenesis in a variety of human malignancies. We aimed to investigate the clinical significance of FOXM1 overexpression in ESCC. Patients and Methods: FOXM1 expression was assessed in ESCC specimens from 174 curatively-resected cases. The relationships between FOXM1 expression, clinicopathological parameters, and prognoses were examined. Results: Immunohistochemical analysis showed that 94 (54.0%) tumors were positive for FOXM1 expression. FOXM1 positivity did not correlate with any clinicopathological parameter. However, FOXM1-positive cases had poorer prognoses than FOXM1-negative ones (p=0.0037, log-rank test). In multivariate analysis, the following were independent prognostic factors: pT, pN, neoadjuvant chemotherapy, and FOXM1 expression (hazard ratio=1.69, 95% confidence interval=1.06-2.75, p=0.027). Conclusion: FOXM1 may be a novel prognostic factor in patients with ESCC who undergo curative resection.*

Esophageal cancer is one of the most aggressive diseases of the gastrointestinal tract (1). In Japan and other East Asian countries, the majority of esophageal cancer diagnoses are esophageal squamous cell carcinoma (ESCC). Despite improvements in surgical technique, chemotherapy, and radiation therapy, the mortality rate of ESCC remains high and its prognosis remains poor because of the high prevalence of invasion and metastasis (2). To improve survival, it is important to identify and characterize tumor-specific molecular markers in ESCC that may contribute to its carcinogenesis.

FOXM1 is a member of the Forkhead family of transcription factors (3, 4). FOXM1 acts in the cell cycle by regulating the transition from the G_1 to the S phase, as well as the progression to mitosis (4-6). FOXM1 is predominantly expressed in fetal tissues, but its expression may be maintained in proliferating adult tissues (5, 6). Overexpression of FOXM1 has been observed in cancer of the liver, breast, prostate, brain, cervix, colon, lung, and stomach (7-14). These findings link FOXM1 to the tumorigenesis and progression of several kinds of malignancies. However, the relationship of FOXM1 to ESCC prognosis remains unclear. In the present study, we investigated whether FOXM1 could be used as an independent biomarker to predict prognosis in patients with ESCC.

Patients and Methods

Patients and treatments. The present study included 174 patients with pathologically-confirmed primary ESCC (Table I) who underwent curative surgical resection at Osaka University Hospital between 2001 and 2007. The study population included 19 women and 155 men; the median age was 64 years (range=46 to 81 years). All patients underwent subtotal esophagectomy *via* right thoracotomy with two- or three-field lymphadenectomy. Non-curative resection was excluded, and curative (R0) resection was achieved for all patients. No patients died of postoperative complications. The 63 patients with lymph node metastasis at initial diagnosis received neoadjuvant chemotherapy (NAC), which consisted of two courses of 5-fluorouracil, cisplatin, and adriamycin. After surgery, patients were surveyed every three months by physical examination and serum tumor markers (squamous cell carcinoma antigen, carcinoembryonic antigen), every six months by computed tomographic scanning and abdominal ultrasonography, and every year by endoscopy until tumor recurrence. Patients with tumor recurrence received chemotherapy or chemoradiotherapy as long as they were able to tolerate it. The mean overall survival (OS) was 46.3 months, and the mean recurrence-free survival (RFS) was 42.8 months.

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Key Words: FOXM1, esophageal squamous cell carcinoma, immunohistochemistry.

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Table I. *Clinical characteristics of 174 patients with esophageal squamous cell carcinoma.*

Parameter	Patients, n (%)
Age, years	64 (46-81)[a]
Gender, male/female	155 (89.0)/19 (11.0)
Histology[b], poor/mod/well	42 (24.1)/93 (53.4)/39 (22.4)
pT[c], 0/1/2/3/4	0 (0)/50 (28.7)/27(15.5)/84 (48.3)/13 (7.5)
pN[c] N0/N1/N2/N3	54 (31.0)/56 (32.2)/37 (21.3)/27 (15.5)
pStage[c] 0/I/II/III/IV	0 (0)/33 (18.9)/41 (23.6)/74 (42.5)/26 (14.9)

[a]Data presented as median (range). [b]Poorly, moderately, and well-differentiated squamous cell carcinoma. [c]pT, pN, pStage (pathological classification) according to the seventh edition of the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification.

Immunohistochemical analysis. FOXM1 expression was evaluated by immunohistochemistry of 4-μm-thick sections of 10% formalin-fixed and paraffin-embedded tissue blocks, as described previously (12). For staining, tissue slides were de-paraffinized in xylene and then rehydrated using graded ethanol. For antigen retrieval, slides were autoclaved in 10 mM citrate buffer (pH 6.0) at 110°C for 20 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 20 min. Non-specific binding was blocked with 10% normal serum for 20 min. Subsequently, tissue slides were incubated overnight with FOXM1 antibody (sc502, dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C in a moist chamber. Sites of antibody binding were visualized with the ABC peroxidase detection system (Vector Laboratories, Burlingame, CA, USA). Finally, sections were incubated in 3,3'-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 for 1 min and counterstained with 0.1% hematoxylin. One representative slide with the deepest tumor invasion was selected from each patient and subjected to immunohistochemistry. The percentage of cancer cells stained with the antibody was then determined. FOXM1 staining for each ESCC sample was defined as positive when more than 10% of the cancer cells in a section were immunoreactive with the FOXM1 antibody; it was defined as negative when 10% or fewer of the cancer cells in a section were positive.

Statistical analysis. Statistical analysis was performed using JMP software (JMP version 9.0.2; SAS Institute, Cary, NC, USA). The relationship between FOXM1 expression and various clinicopathological parameters was assessed using the χ^2 test. RFS and OS were assessed using the Kaplan–Meier method and compared using the log-rank test. All parameters found to be significant in univariate analysis using the Cox proportional hazards model were entered into multivariate survival analysis. *p*-Values <0.05 were considered significant; each *p*-value was derived from a two-tailed test.

Results

FOXM1 expression in ESCC. A total of 174 samples (Table I) that contained both cancerous and non-cancerous lesions were evaluated for FOXM1 expression by immunohistochemistry. Out of these, 94 (54.0%) were positive for FOXM1 expression; staining was mainly cytoplasmic, with faint nuclear staining in tumor cells (Figure 1A). The remaining 80 (46.0%) samples were negative for FOXM1 expression (Figure 1B). In contrast, none of the samples of normal squamous epithelium exhibited substantial FOXM1 staining, although some basal cells exhibited faint nuclear immunostaining (Figure 1C). FOXM1-positive cells were detected in various parts of the tumors, including the surface, central, and deep areas of the esophagus.

Table II. *Correlation between FOXM1 expression and clinicopathological parameters.*

Parameters	FOXM1 expression		
	Positive (%)	Negative (%)	*p*-Value
Age, years			
<65	46 (26.4)	43 (24.7)	0.53
≥65	48 (27.6)	37 (21.3)	
Gender			
Male	84 (48.3)	71 (40.8)	0.90
Female	10 (5.8)	9 (5.2)	
Histology[a]			
Poor, moderate	74 (42.5)	61 (35.1)	0.70
Well	20 (11.5)	19 (10.9)	
Neoadjuvant chemotherapy			
Yes	35 (20.1)	28 (16.1)	0.76
No	59 (33.9)	52 (29.9)	
pT[b]			
T1-2	38 (21.8)	39 (22.4)	0.27
T3-4	56 (32.2)	41 (23.6)	
pN[b]			
N0	24 (13.8)	30 (17.2)	0.089
N1-3	70 (40.2)	50 (28.4)	
pStage[b]			
I, II	36 (20.7)	38 (21.8)	0.22
III, IV	58 (33.3)	42 (24.1)	

[a]Poorly, moderately, and well-differentiated squamous cell carcinoma. [b]pN, pT, pStage (pathological classification) according to the seventh edition of the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification.

Correlation between FOXM1 expression and clinicopathological parameters. Table II lists the correlations between FOXM1 expression and various clinicopathological parameters. No significant correlations were observed between FOXM1 expression and other parameters, including age, sex, histology, use of NAC, or depth of tumor invasion (Table II).

Correlation between FOXM1 expression and survival. The total 5-year OS rate was 52.7%. Patients with FOXM1-positive tumors exhibited poorer OS than those with negative tumors (5-year OS 42.8% *versus* 64.8%, p=0.0037; Figure 2A). Similarly, patients with FOXM1-positive tumors exhibited poorer 5-year RFS than those with FOXM1-

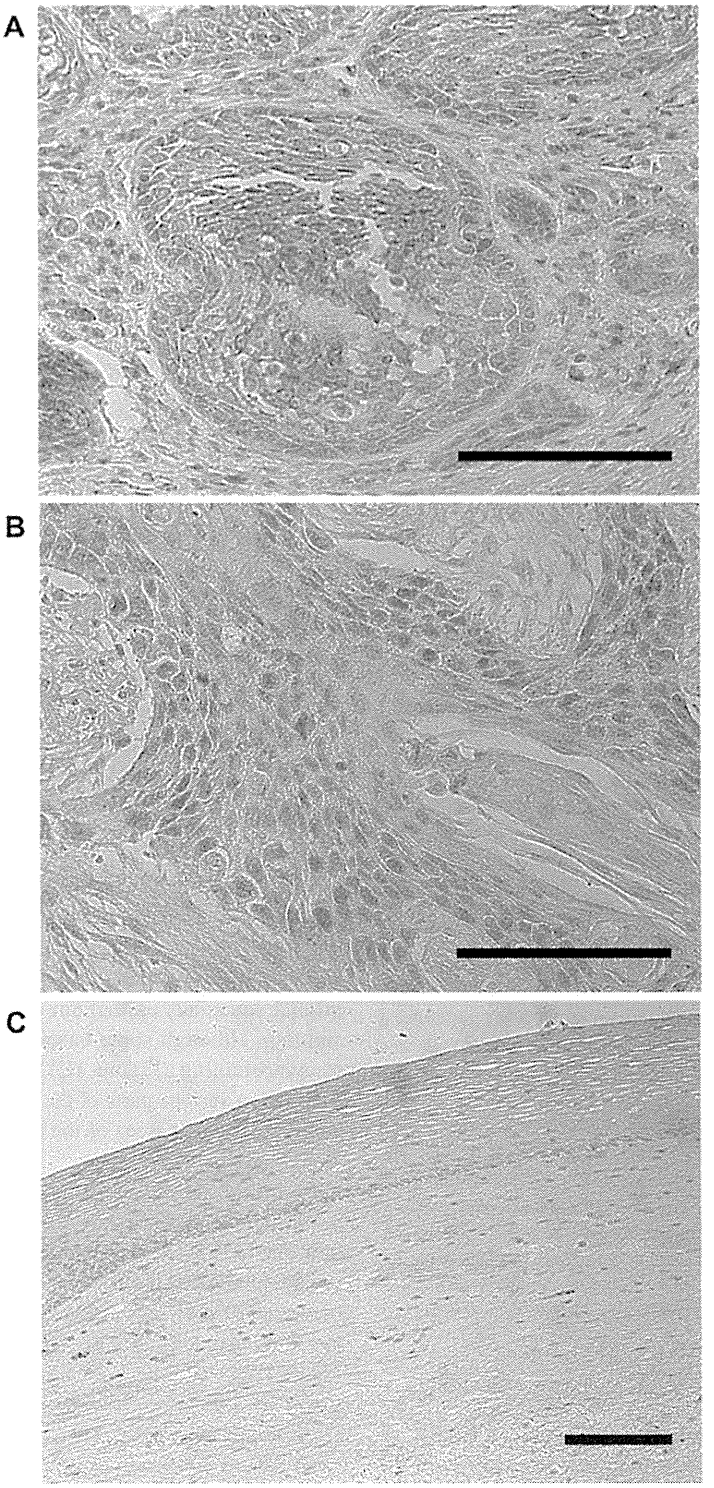

Figure 1. *FOXM1 expression determined by immunohistochemical staining. A: Representative FOXM1-positive esophageal squamous cell carcinoma exhibiting staining mainly in the cytoplasm of tumor cells (magnification ×200). B: Representative FOXM1-negative esophageal squamous cell carcinoma exhibiting almost no staining of tumor cells (magnification ×200). C: Representative normal squamous epithelium that was negative for FOXM1 expression except in a few basal cells (magnification ×100). Scale bars, 100 μm.*

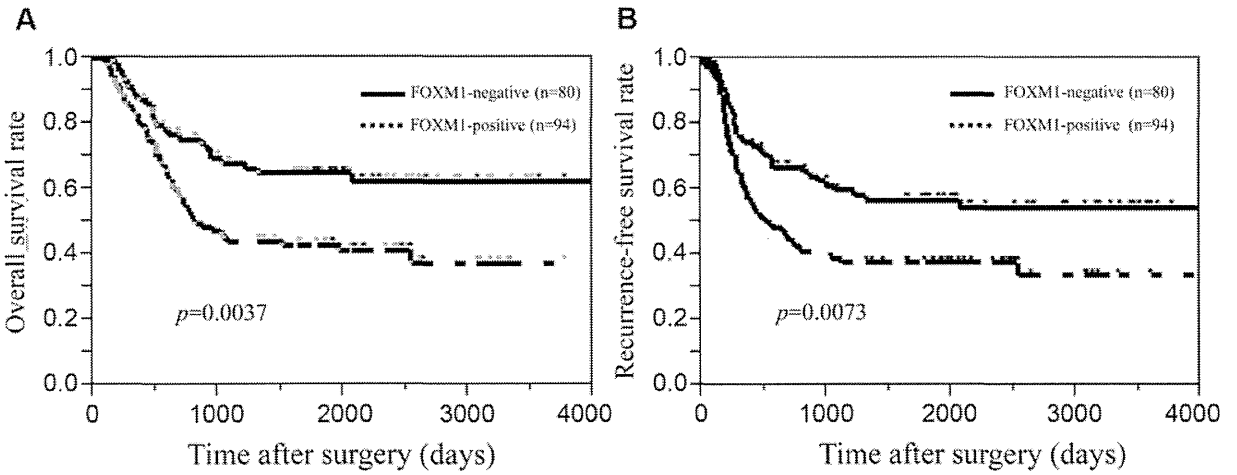

Figure 2. *Survival curves according to FOXM1 expression. A: Overall survival curve according to FOXM1 expression for all patients plotted by the Kaplan–Meier method. B: Recurrence-free survival curves according to FOXM1 expression for all patients. Differences between the two groups were evaluated using the log-rank test.*

Table III. *Univariate and multivariate analysis of overall survival using Cox's proportional hazard model.*

Parameter	Number of cases	Univariate		Multivariate	
		HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Age, ≥65 years *vs.* < 65 years	89 *vs.* 85	1.13 (0.73-1.76)	0.57		
Sex, female *vs.* male	19 *vs.* 155	1.02 (0.47-1.93)	0.96		
Histology, poor, moderate *vs.* well[a]	135 *vs.* 39	1.54 (1.87-2.91)	0.14		
pT (T3, 4 *vs.* T1, 2)[b]	97 *vs.* 77	2.48 (1.56-4.05)	<0.0001	1.69 (1.04-2.82)	0.033
pN (N1-3, N0)[b]	120 *vs.* 54	3.56 (2.01-6.93)	<0.0001	2.77 (1.54-5.42)	0.0004
Neoadjuvant chemotherapy, yes *vs.* no	63 *vs.* 111	2.36 (1.52-3.66)	0.0001	1.97 (1.26-3.10)	0.0031
FOXM1 expression, positive *vs.* negative	94 *vs.* 80	1.95 (1.24-3.15)	0.0034	1.69 (1.06-2.75)	0.027

[a]Poorly, moderately, and well differentiated squamous cell carcinoma. [b]pT, pN, (pathological classification) according to the seventh edition of the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification. HR: Hazard ratio; CI: confidence interval.

negative tumors. In univariate analysis, the following were significantly associated with OS: pT [hazard ratio (HR)=2.48, 95% confidence interval (CI)=1.56-4.05, $p<0.0001$], pN (HR=3.56, 95% CI=2.01-6.93, $p<0.0001$), NAC (HR=2.36, 95% CI=1.52-3.66, $p=0.0001$), and FOXM1 expression (HR=1.95, 95% CI=1.24-3.15, $p=0.0034$) (Table III). The four parameters that showed statistical significance ($p<0.05$) in univariate analysis were entered into multivariate analysis. Multivariate analysis revealed that pN was the poorest prognostic factor (HR=2.77, 95% CI=1.54-5.42, $p=0.0004$), followed by NAC (HR=1.97, 95% CI=1.26-3.10, $p=0.0031$), pT (HR=1.69, 95% CI=1.04-2.82, $p=0.033$), and positive FOXM1 expression (HR=1.69, 95% CI=1.06-2.75, $p=0.027$) (Table III).

Discussion

In the present study, we investigated the expression of FOXM1 in ESCC tissues. To our knowledge, this is the largest series of samples analyzed for FOXM1 expression in ESCC to date. Our analysis revealed that FOXM1 expression in ESCC is an independent prognostic indicator for OS. This finding is consistent with previous reports (7, 11, 12, 15, 16). In our series, patients with advanced ESCC received NAC. Thus, NAC became a strong prognostic factor for OS. As far as we are aware, there is just one report on the association between FOXM1 and ESCC in clinical samples (17). In that study, Hui *et al.* reported that FOXM1 overexpression was associated with pathological stage, but not with prognosis of patients with

ESCC. However, it might be premature to conclude that FOXM1 is not associated with the prognosis of patients with ESCC. The report by Hui *et al*., assessed only 64 patients, and may have been too small to reveal an association between FOXM1 expression and prognosis. Notably, although that study did not find an association between FOXM1 expression and prognosis, it did show a positive association between FOXM1 expression and pathological stage.

FOXM1 is a proliferation-associated transcription factor with important roles in cell proliferation, differentiation, and apoptosis (5, 6, 18). However, the mechanism by which FOXM1 signaling induces tumor growth is not well-understood. Multiple pathways crosstalk with the FOXM1 pathway, including the phosphatidylinositol 3-kinase/protein kinase B (Akt) (19, 20), nuclear factor-κB (21), sonic hedgehog (22), extracellular signal-regulated kinase (23), cyclooxygenase-2 (24), epidermal growth factor receptor (25, 26), vascular endothelial growth factor (27, 28), avian myelocytomatosis virus oncogene cellular homolog (c-MYC) (29, 30), p53 (31, 32), and hypoxia-inducible factor-1 pathways (33). Thus, these reports strongly suggest that FOXM1 is centrally-involved in tumor aggressiveness. In our analysis FOXM1 expression was associated not only with OS but also RFS, this phenomenon was consistent with these mechanisms.

Overexpression of FOXM1 in tumor cell lines is correlated with resistance to apoptosis and to premature senescence induced by oxidative stress, which is strongly implicated in resistance to chemotherapy (34). Recent studies show that FOXM1 is overexpressed in a variety of human cancer types and is crucially-implicated in tumorigenesis (3, 8-10, 35, 36). Furthermore, down-regulation of FOXM1 leads to inhibition of cell growth, migration, and invasion in several cancer types (36-38). These results suggest that FOXM1 may play a crucial role in the development and progression of human cancer. Therefore, although more studies are required, inactivation of FOXM1 may represent a promising strategy for developing novel and selective anticancer therapies.

In conclusion, here we examined the expression of FOXM1 protein in ESCC specimens and investigated correlations between FOXM1 overexpression and clinicopathological characteristics. Patients that were positive for FOXM1 expression had worse prognoses. Thus, evaluation of FOXM1 expression might help identify a subset of patients with ESCC who need more intensive treatment.

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Expression of insulin-like growth factor-II mRNA-binding protein-3 as a marker for predicting clinical outcome in patients with esophageal squamous cell carcinoma

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Abstract. Insulin-like growth factor-II mRNA-binding protein-3 (IMP3) is an important factor in carcinogenesis, although its clinical significance in esophageal squamous cell carcinoma (ESCC) remains unknown. The present study investigated the associations between IMP3 expression and the clinicopathological parameters. IMP3 expression was assessed in 191 resected ESCC specimens, and the associations between IMP3 expression in ESCC, the clinicopathological parameters and patient prognosis were examined. Using immunohistochemistry, 113 (59.2%) tumors were identified as IMP3-positive. IMP3 positivity correlated significantly with high pathological (p)Stage, pT stage and pN stage. The IMP3-positive patients exhibited a poorer prognosis compared with the IMP3-negative patients. In univariate analyses, histology [hazard ratio (HR), 1.94; 95% confidence interval (CI), 1.18-3.49; P=0.0082], pT (HR, 2.34; 95% CI, 1.55-3.62; P<0.0001), pN (HR, 2.85; 95% CI, 1.81-4.69; P<0.0001), lymphatic invasion (HR, 2.08; 95% CI, 1.26-3.70; P=0.0036), venous invasion (HR, 1.79; 95% CI, 1.21-2.64; P=0.0039), neoadjuvant chemotherapy (NAC) (HR, 2.01; 95% CI, 1.35-3.00; P=0.0005) and IMP3 expression (HR, 2.12; 95% CI, 1.40-3.29; P=0.0003) were significantly associated with overall survival. Using multivariate analyses, histology (HR, 1.87; 95% CI, 1.13-3.29; P=0.014), pN (HR, 2.19; 95% CI, 1.36-3.66; P=0.0010), NAC (HR, 1.88; 95% CI, 1.24-2.86; P=0.0028) and IMP3 expression (HR, 1.84; 95% CI, 1.18-2.93; P=0.0064) were significant prognostic factors. IMP3 may therefore be a prognostic factor for patients with ESCC who have undergone a curative resection.

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Key words: insulin-like growth factor-II mRNA-binding protein-3, esophageal squamous cell carcinoma, immunohistochemistry

Introduction

In East Asian countries, esophageal squamous cell carcinoma (ESCC) is the major histological form of esophageal cancer. The disease is also one of the most lethal digestive tract malignances (1). In the majority of cases, the initial diagnosis of ESCC is made when the malignancy has already progressed to an advanced stage (1). Despite recent improvements in multi-treatment approaches, including surgery, radiotherapy and chemotherapy, the prognosis for patients with ESCC remains unsatisfactory (2). Predicting a prognosis by examining the clinicopathological characteristics remains difficult, even when using the tumor-node-metastasis staging system. This is due to considerable tumor variability and heterogeneity within the same pathological stage.

The IMP3 gene, also known as the K homology domain-containing gene (KOC) or L523S, encodes the IMP3 protein (3). IMP3 is located on chromosome 7p11.5 and encodes a 4350-bp mRNA and a 580-aa protein. IMP3 is expressed in the developing epithelium, muscle and placenta during the early stages of human and mouse embryogenesis, and low or undetectable levels of IMP3 are present in adult tissues (4,5). IMP3 has been shown to be overexpressed in testicular cancer, renal cell carcinoma, ovarian carcinoma, gastric cancer, colon cancer and adenocarcinoma of the lung (6-15). The IMP3 protein, together with IMP-1 and IMP-2, has different functions in various post-transcriptional processes, including mRNA localization, mRNA turnover and translational control (16-19). The IMP3 gene has previously been used as a marker to detect malignant cells in fine-needle aspirates (20). Additionally, in K562 leukemia cells, the inhibition of IMP3 has been shown to result in apoptosis, indicating that it may be vital for cancer cell survival (18). IMP3 is a prognostic biomarker in patients with endometrial serous carcinoma and renal cell carcinoma. In such cases, IMP3 expression appears to predict an increased likelihood of metastasis following surgery and a shorter metastasis-free survival time (8-11,15). However, to the best of our knowledge, the clinicopathological and prognostic significance of IMP3 expression in ESCC remains unknown. In the present study, the prevalence and clinicopathological significance of IMP3 expression were investigated with regard

to overall survival (OS) and recurrence-free survival (RFS) in 191 patients.

Materials and methods

Patients and treatments. The present study examined 191 patients with pathologically confirmed primary ESCC who underwent surgical resection at the Osaka University Hospital (Osaka, Japan) between 1998 and 2007 (Table I). Approval for the study was obtained from the Ethics Committee of Osaka University Hospital. The study population consisted of 24 female and 167 male patients who ranged between 29 and 85 years of age (median, 62.7 years). All patients underwent a subtotal esophagectomy via a right thoracotomy, with a two- or three-field lymphadenectomy, with curative resection. None of the patients succumbed to post-operative complications. Of the 104 patients with lymph node metastases at the initial diagnosis, 86 received neoadjuvant chemotherapy (NAC), which consisted of two courses of 5-fluorouracil (700 mg/m^2 on days one to seven), cisplatin (70 mg/m^2 on day one) and Adriamycin (35 mg/m^2 on day one). Following surgery, the patients were followed up every 3 months by physical examination and an analysis of serum tumor markers (squamous cell carcinoma antigen and carcinoembryonic antigen), every 6 months by computed tomography scanning and abdominal ultrasonography, and every year by endoscopy until tumor recurrence became evident. Patients exhibiting tumor recurrence received chemotherapy or chemoradiotherapy for as long as this regimen was systemically tolerated. The mean OS time was 41 months, and the mean RFS time was 39 months.

Immunohistochemical analysis. IMP3 expression was examined in formalin-fixed, paraffin-embedded ESCC tissue sections by immunohistochemistry (IHC). One representative slide with the deepest tumor invasion was selected from each patient and examined by IHC. The tissue sections were deparaffinized in xylene and then rehydrated through a graded ethanol series. For antigen retrieval, the slides were incubated by autoclaving at 110˚C in 10 mm Tris and 1 mm EDTA buffer (pH 9.0) for 20 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 20 min and non-specific binding was blocked with 10% normal serum for 20 min. Subsequently, the tissue slides were incubated overnight with anti-IMP3 antibody (monoclonal mouse anti-human L523S; dilution, 1:200; Dako Cytomation, Carpinteria, CA, USA) at 4˚C in a humidified chamber. The bound antibody was visualized using the Avidin/Biotin Complex Peroxidase Detection System (Vector Laboratories, Burlingame, CA, USA). Finally, the sections were incubated in 3,3'-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 for 3 min and counterstained with 0.1% hematoxylin. IMP3 staining for each ESCC sample was defined as positive when >10% of the cancer cells in the section were immunoreactive with the anti-IMP3 antibody. Staining was defined as negative when ≤10% of the cancer cells in the section were positive.

Statistical analysis. Statistical analysis was performed using JMP software (JMP version 9.0.2; SAS Institute, Cary, NC, USA). The association between IMP3 expression and the clinicopathological parameters was assessed using the χ^2 test. The RFS and OS were assessed using the Kaplan-Meier method and compared using the log-rank test. All the parameters that were found to be significant in a univariate analysis using the Cox proportional hazard model were entered into a multivariate survival analysis. P-values were derived from two-tailed testing and $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Characteristics of patients with ESCC.

Parameters	Value
Median age, years (range)	62.7 (29-85)
Gender, n (%)	
Male	167 (87.4)
Female	24 (12.6)
Histology of SCC, n (%)	
Poorly-differentiated	45 (23.6)
Moderately-differentiated	99 (51.8)
Well-differentiated	47 (24.6)
Pathological classification[a], n (%)	
pT	
0	0 (0.0)
1	51 (26.7)
2	30 (15.7)
3	93 (48.7)
4	17 (8.9)
pN	
N0	68 (35.6)
N1	53 (27.7)
N2	35 (18.3)
N3	35 (18.3)
pStage	
0	0 (0.0)
I	39 (20.4)
II	53 (27.7)
III	63 (33.0)
IV	36 (18.8)

[a]According to the Union for International Cancer Control, 7th edition (21). ESCC; esophageal squamous cell carcinoma; pN; pathological N stage; pT, pathological T stage; pStage, pathological stage.

Results

IMP3 expression in ESCC. A total of 191 samples that contained cancerous and non-cancerous lesions were evaluated for IMP3 expression using IHC. Of these, 113 (59.2%) showed positive IMP3 expression that was predominantly localized to the cytoplasm of the tumor cells, along with faint nuclear staining (Fig. 1A). The remaining 78 (40.8%) were negative for

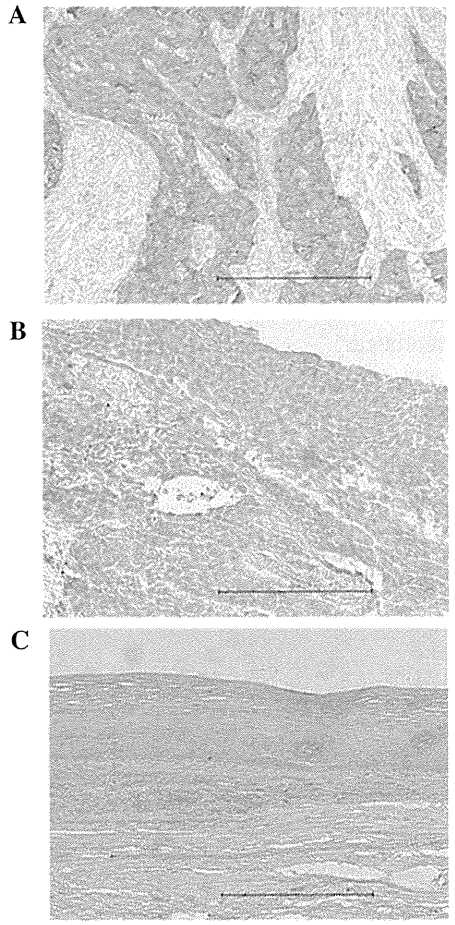

Figure 1. Representative images of IMP-3 expression, as determined by immunohistochemical staining. (A) IMP-3-positive esophageal squamous cell carcinoma exhibiting staining mainly in the cytoplasm of the tumor cells. (B) IMP-3-negative esophageal squamous cell carcinoma exhibiting almost no staining of the tumor cells. (C) Normal squamous epithelium negative for IMP-3. The black scale bar represents 250 μM. IMP3, insulin-like growth factor-II mRNA-binding protein-3.

IMP3 expression (Fig. 1B). The positive staining was almost homogeneous in individual cancer foci and in different areas, such as in the surface, central and deepest areas, of the cancer lesions. By contrast, none of the normal squamous epithelia exhibited substantial IMP3 staining, although certain basal cells showed faint nuclear staining (Fig. 1C).

Association between IMP3 expression and clinicopathological parameters. Table II lists the associations between IMP3 expression and the clinicopathological parameters. The IMP3-positive tumors were significantly associated with deeper tumor invasion and lymph node metastases compared with the IMP3-negative tumors (P=0.0001 and P=0.026, respectively). No significant associations were observed between IMP3 expression and other parameters, including age, gender, histology and use of NAC.

Association between IMP3 expression and survival. The 5-year OS rate of the population was 48.5%. Patients with IMP3-positive tumors experienced a poorer 5-year OS rate compared with those with IMP3-negative tumors (39.3 vs. 61.7%, P=0.0004; Fig. 2A). Similarly, patients with IMP3-positive tumors experienced a poorer RFS rate compared with those with IMP3-negative tumors (35.7 vs. 61.9%, P=0.0004; Fig. 2B). By univariate analyses, histology [hazard ratio (HR), 1.94; 95% confidence interval (CI), 1.18-3.49; P=0.0082], pathological T stage (pT; HR, 2.34; 95% CI, 1.55-3.62; P<0.0001), pathological N stage (pN; HR, 2.85; 95% CI, 1.81-4.69; P<0.0001), lymphatic invasion (HR, 2.08; 95% CI, 1.26-3.70; P=0.0036), venous invasion (HR, 1.79; 95% CI, 1.21-2.64; P=0.0039), NAC (HR, 2.01; 95% CI, 1.35-3.00; P=0.0005), and IMP expression (HR, 2.12; 95% CI, 1.40-3.29; P=0.0003) were significantly correlated with OS (Table III). The seven parameters that demonstrated statistical significance (P<0.05) by univariate analysis were further analyzed by multivariate analysis. Multivariate analysis showed that pathological lymph node metastasis was the poorest prognostic factor (HR, 2.19; 95% CI, 1.36-3.66; P=0.0010), followed by NAC (HR, 1.88;

Table II. Correlation between IMP3 expression and clinicopathological parameters.

	IMP3 expression, n (%)		
Parameters	Positive	Negative	P-value
Age, years			
<65	64 (33.5)	47 (24.6)	0.6179
≥65	49 (25.7)	31 (16.2)	
Gender			
Male	97 (50.8)	70 (36.6)	0.4191
Female	16 (8.4)	8 (4.2)	
Histology[a]			
Poor/moderate	89 (46.6)	55 (28.8)	0.1955
Well	24 (12.6)	23 (12.0)	
Neoadjuvant chemotherapy			
Yes	48 (25.1)	38 (19.9)	0.4654
No	65 (34.0)	40 (20.9)	
Depth of tumor invasion[b]			
pT1-2	35 (18.3)	46 (24.1)	0.0010
pT3-4	78 (40.8)	32 (16.8)	
Lymph node metastasis[b]			
pN0	33 (17.3)	35 (18.3)	0.0267
pN1-3	80 (41.9)	43 (22.5)	
pStage[b]			
I, II	67 (35.1)	46 (24.1)	0.0003
III, IV	46 (24.1)	32 (16.8)	

[a]Well-, moderately- and poorly-differentiated squamous cell carcinoma. [b]According to the Union for International Cancer Control, 7th edition (21). pN; pathological N stage; pT, pathological T stage; pStage, pathological stage; IMP3, insulin-like growth factor-II mRNA-binding protein-3.

Table III. Univariate and multivariate analysis of OS using Cox's proportional hazard model.

Parameter	Number of cases	Univariate		Multivariate	
		HR (95% CI)	P-value	HR (95% CI)	P-value
Age (>65 years)	78/113	1.24 (0.84-1.84)	0.2766		
Gender (female/male)	24/167	1.05 (0.56-1.82)	0.8591		
Histology (poor-moderate/well)[a]	144/47	1.94 (1.18-3.49)	0.0082	1.87 (1.13-3.29)	0.0134
pT (T3,4/T1,2)[b]	110/81	2.34 (1.55-3.62)	<0.0001	1.28 (0.79-2.10)	0.3303
pN (N1-3, N0)[b]	123/68	2.85 (1.81-4.69)	<0.0001	2.19 (1.36-3.66)	0.0010
Lympathic invasion (present/absent)	148/43	2.08 (1.26-3.70)	0.0036	1.11 (0.62-2.08)	0.7354
Venous invasion (present/absent)	79/112	1.79 (1.21-2.64)	0.0039	1.22 (0.79-1.91)	0.3740
NAC (yes/no)	86/105	2.01 (1.35-3.00)	0.0005	1.88 (1.24-2.86)	0.0028
IMP3 expression (positive/negative)	113/78	2.12 (1.40-3.29)	0.0003	1.84 (1.18-2.93)	0.0064

[a]Well-, moderately- and poorly-differentiated squamous cell carcinoma. [b]According to the Union for International Cancer Control, 7th edition (21). OS, overall survival; pN; pathological N stage; pT, pathological T stage; HR, hazard ratio; CI, confidence interval; IMP3, insulin-like growth factor-II mRNA-binding protein-3; NAC, neoadjuvant chemotherapy.

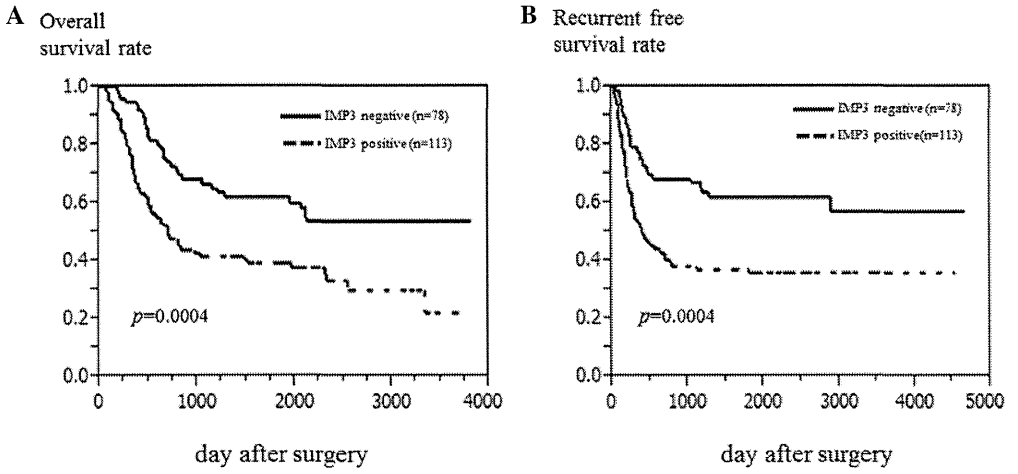

Figure 2. Survival curves according to IMP-3 expression. (A) Overall survival of all patients was plotted using the Kaplan-Meier method. (B) Recurrence-free survival of all patients. IMP3, insulin-like growth factor-II mRNA-binding protein-3.

95% CI, 1.24-2.86; P=0.0028), histology (HR, 1.87; 95% CI, 1.13-3.49; P=0.014), and IMP3 expression (HR, 1.84; 95% CI, 1.18-2.93; P=0.0064) (Table III).

Discussion

IMP3 is an RNA-binding protein and a KH domain-containing member of the IMP family. In mice, IMPs are primarily expressed during early embryogenesis and at mid-gestation, but they are not expressed in the majority of adult human tissues (3,4,22). IMP3 has been reported to function by regulating tumor cell proliferation, migration and metastasis. IMP3 has been shown to promote tumor cell proliferation through the upregulation of IGF2, a potent mitogenic factor previously shown to exert effects in a number of diseases (18,23,24). Studies have additionally found that IMP3 can exert a marked effect on cellular adhesion and invasion during normal development and during the development of cancers (25). For these reasons, strong IMP3 expression is regarded as an indicator of a poor prognosis (6,9,10,26,27). However, to the best of our knowledge, the clinicopathological and prognostic significance of IMP3 expression in ESCC has not been reported.

The present study demonstrated the positive immunoreactivity to IMP3 of 59.2% of ESCC surgical samples. Positive IMP3 expression was significantly associated with pathological factors associated with tumor progression [pT, pN and pathological stage (pStage)]. IMP3 was identified as a prognostic factor for OS. Although pT is generally considered to be an independent prognostic factor, this was not the case in the present series. In the present study, patients with advanced ESCC received NAC. Hence, the effect of pT was canceled by the effect of NAC in the multivariate analysis. This result was similar to that reported in other cancers (6,9-11,26,27). However, the clinical association between IMP3 and a worse prognosis of ESCC remains poorly defined. Yoshino *et al* (28) reported that IMP3 mRNA expression was associated with

resistance to radiation therapy in ESCC cell lines. Further studies to investigate this should therefore be performed in the future.

Several characteristics of IMP3 indicate that it may be a potentially attractive prognostic marker. First, IMP3 IHC staining is a simple and reliable assay to perform (9). In the majority of cases, carcinomas are treated surgically, allowing chemotherapy and radiation therapy to be combined. Tumor tissues are thus routinely available for IHC staining using the monoclonal L523 antibody. The present study found that IMP3 IHC was reproducible and could be readily performed on ESCC tissues. The simplicity of this assay will enable a pre-operative diagnosis from the analysis of biopsy tissue. Regarding the polymerase chain reaction (PCR)-based method, IMP3 has been used as a molecular marker to predict peritoneal recurrence following curative surgery for gastric cancer (11), and PCR amplification of IMP3 from biliary structure specimens have been useful to distinguish between benign and malignant lesions (29). Furthermore, IMP3 has been considered a potential target for immunotherapy. A phase II study using a peptide vaccine therapy, which included IMP3, has been performed for patients with advance ESCC who failed to respond to standard therapies (30). It has been reported that the immune response induced by the vaccination may improve the prognosis for patients with advanced ESCC.

In conclusion, in the present study, IMP3, a novel mRNA-binding protein, was shown to be frequently expressed in ESCC. IMP3 expression was more commonly observed in ESCC patients with poor prognostic factors. IMP3 may be a potential IHC biomarker that can be used to evaluate the tumor progression and prognosis of ESCC.

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ORIGINAL ARTICLE

Single-incision laparoscopic partial gastrectomy for gastric submucosal tumors without compromising transumbilical stapling

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Abstract

Introduction: Although SILS has become an increasingly popular type of surgery, its application for gastric submucosal tumors (SMT) has been only sporadically reported. We herein describe 12 recent cases with gastric SMT located in the greater curvature or anterior wall. The aim is to validate technical feasibility and safety of single-incision laparoscopic partial gastrectomy. Thus far, this is one of the largest series of patients with gastric SMT who underwent SILS.
Methods: From July 2009 to April 2013, single-incision laparoscopic partial gastrectomy was attempted in 12 consecutive patients with gastric SMT. Three trocars were assembled in the umbilical incision, and the lesion was mobilized and staple-resected with endoscopic stapling devices.
Results: SILS surgery was successfully completed without any additional trocars. The median operating time was 96.5 min, and median blood loss was 7.5 mL. The median tumor size was 30 mm, with histopathologic diagnosis of gastrointestinal stromal tumor (10) and schwannoma (2). There was no immediate postoperative morbidity. During a median follow-up of 12 months, all patients were on full regular diet without any gastrointestinal symptoms.
Conclusion: SILS with transumbilical gastric stapling is a safe and practical alternative to conventional multiport laparoscopy in patients with gastric SMT, except for cases originating in the lesser curvature and close to the cardia/ pylorus.

Introduction

With the recent improvements in instrumentation and procedures, SILS has become increasingly popular for various gastrointestinal procedures (1–3). Theoretically, gastric submucosal tumors (SMT) are one of the best candidates for SILS, as partial gastrectomy for gastric SMT is a relatively simple procedure that requires no lymph node dissection (4,5). However, SILS only offers a limited range of motion. For example, particularly when the stapling device is inserted via the umbilicus, stapling becomes complicated because of the device's limited handling among the crowded transumbilical instruments.

Despite the potential that SILS offers, few reports are available in the surgical literature (6,7), and the role of SILS in the surgical management of gastric SMT is not yet fully understood. The aim of this study was to evaluate the feasibility and safety of SILS partial gastrectomy for gastric SMT, with technical considerations including specimen retrieval and application of transumbilical gastric stapling. To our knowledge, this is one of the largest series of patients with gastric SMT who underwent SILS.

Table 1 Preoperative characteristics of 12 SILS for gastric SMT at a single institution (July 2009–April 2013)

				Preoperative diagnosis			
Case no.	Age (years)	Gender	BMI (kg/m^2)	Growth appearance	Location		Tumor (mm)
1	49	Male	22.4	Exogastric	Middle	Greater curvature	30
2	55	Male	19.1	Exogastric	Middle	Anterior wall	25
3	60	Male	25.9	Exogastric	Upper	Greater curvature	30
4	69	Male	22.7	Intramural	Middle	Anterior wall	25
5	65	Male	17.8	Exogastric	Upper	Anterior wall	20
6	54	Male	20.7	Exogastric	Upper	Greater curvature	30
7	60	Male	25.4	Exogastric	Upper	Greater curvature	35
8	63	Male	25.0	Exogastric	Upper	Greater curvature	30
9	50	Female	21.5	Exogastric	Middle	Posterior wall	24
10	64	Male	22.5	Intramural	Upper	Greater curvature	28
11	47	Male	15.6	Exogastric	Middle	Anterior wall	35
12	80	Male	22.4	Exogastric	Middle	Greater curvature	30

SMT, submucosal tumor.

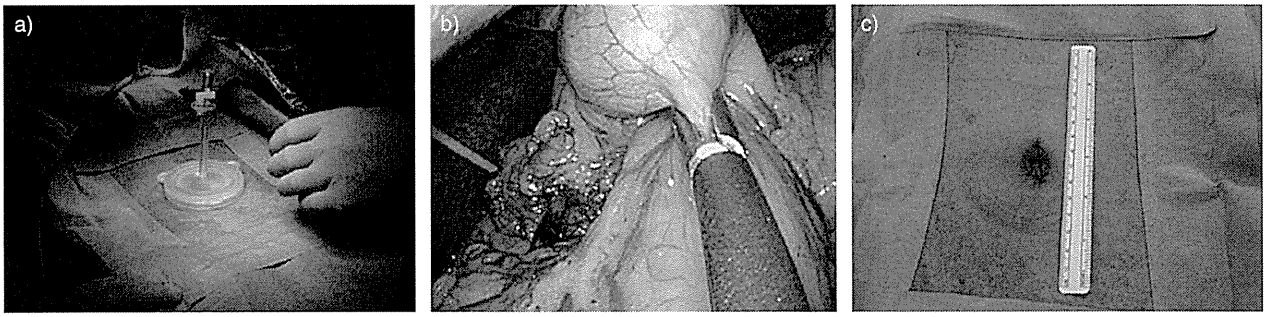

Figure 1 (a) An infraumbilical incision was made by pulling out the umbilicus. The peritoneum was incised, an EZ Access port (HAKKO) was inserted, and 5-mm trocars were then inserted through the port. (b) The stomach was clamped on the resection line, and intraoperative endoscopy was simultaneously performed. (c) The wound was closed with absorbable sutures.

Materials and Methods

Patients

For 12 consecutive patients with gastric SMT, SILS partial gastrectomy was offered as an alternative to conventional multiport laparoscopic partial gastrectomy between July 2009 and April 2013. All patients met our inclusion criteria for SILS partial gastrectomy: (i) tumor size less than 5 cm; (ii) exogastric/intramural tumor growth; (iii) no lesser curvature involvement; and (iv) lesions not adjacent to the cardia or pylorus. There were 11 men and 1 woman, with a median age of 60 years (Table 1). All patients underwent preoperative work-up using esophagogastroduodenoscopy and CT, which confirmed size, location and growth pattern of the tumors. Fine-needle aspiration cytology was performed on two patients with preoperative diagnosis of gastrointestinal stromal tumor (GIST).

Surgical technique

A single, vertical, 25-mm transumbilical incision was made by pulling out the umbilicus. A commercially available access device for SILS (EZ Access, HAKKO, Nagano, Japan) was assembled, and carbon dioxide pneumoperitoneum was created, with intra-abdominal pressure of 12 mmHg (Figure 1a). Three 5-mm trocars were used for the flexible 5-mm laparoscope (LTF TYPE VP, Olympus Medical Systems, Tokyo, Japan) and laparoscopic graspers.

After the tumor was located, the greater omentum was divided using ultrasonic coagulating shears. We always performed wide mobilization while retracting the stomach around the tumor without grasping the tumor itself. The resection line was designed by provisional clamping (Figure 1b). At this point, intraoperative endoscopy was performed to exclude gastric passage distur-

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Table 2 Postperative characteristics of 10 SILS for gastric SMT at a single institution (July 2009–April 2013)

Case no.	Growth appearance	Location		Tumor (mm)	Operating time (min)	Blood loss (mL)	Pathological diagnosis	Ancillary use
1	Exogastric	Middle	Greater curvature	30 × 25 × 25	65.0	10.0	GIST	None
2	Exogastric	Middle	Anterior wall	27 × 25 × 23	59.0	Neg	GIST	None
3	Exogastric	Upper	Greater curvature	31 × 25 × 23	110.0	Neg	Schwannoma	Done
4	Intramural	Middle	Anterior wall	25 × 20 × 20	102.0	Neg	GIST	Done
5	Exogastric	Upper	Anterior wall	18 × 16 × 15	57.0	Neg	GIST	None
6	Exogastric	Upper	Greater curvature	30 × 25 × 17	134.0	10.0	GIST	Done
7	Exogastric	Upper	Greater curvature	38 × 35 × 28	123.0	30.0	GIST	Done
8	Exogastric	Upper	Greater curvature	30 × 24 × 19	104.0	Neg	GIST	Done
9	Exogastric	Middle	Posterior wall	24 × 20 × 18	129.0	15.0	Schwannoma	None
10	Intragastric	Upper	Greater curvature	28 × 22 × 15	91.0	10.0	GIST	Done
11	Exogastric	Middle	Anterior wall	36 × 25 × 15	66.0	5.0	GIST	Done
12	Exogastric	Middle	Greater curvature	30 × 25 × 25	83.0	10.0	GIST	None

GIST, gastrointestinal stromal tumor; neg, negligible amount; SMT, submucosal tumor.

bance and/or any extreme deformity of the gastric remnant. One of the 5-mm trocars was then exchanged for a 12-mm trocar, and stapled-resection was performed using endoscopic linear staplers. The specimen was isolated in a specimen bag and retrieved via the umbilical wound. The wound was closed with absorbable sutures (Figure 1c).

Results

Table 2 depicts the surgical results. SILS partial gastrectomy was completed in all patients without addition of ports. In 7 of 12 cases, we elevated the left lateral segment of the liver with a 2-mm loop-type retracting device (Mini-Loop Retractor II, Covidien, Norwalk, USA) to fully expose the lesion. Median operating time was 96.5 min (range, 57.0–134.0 min), and the median blood loss was 7.5 mL (range, 0.0–30.0 mL). The median tumor size was 30 mm (18–38 mm). In all cases, the postoperative course was rapid and uneventful.

For 10 of 12 patients, gastric GIST was confirmed by immunohistochemistry. In all patients, the margins were free of disease. According to Fletcher's classification, there was 1 patient with "very low risk," 10 with "low risk," and 1 patient with "intermediate risk." Two cases of gastric schwannoma were also confirmed. During a median follow-up of 12 months (range, 1–41 months), there were neither tumor recurrences nor metastases. Although one patient continued to need H_2 receptor antagonist to resolve his preexisting reflux symptom, other patients had no postoperative complaints, such as anorexia, dyspepsia, or epigastric discomfort. On esophagogastroduodenoscopy 1-year after surgery (eight patients), there was no food residue and/or bile reflux in the remnant stomach. The function of the gastric remnant was considered well preserved in this series.

Discussion

Complete gross tumor resection with preservation of organ function is a standard treatment for gastric GIST (8–12). Because GIST usually grows out from the primary organ instead of being diffusely infiltrating, the procedure does not require wide negative margins. In addition, lymph node dissection is not necessary because GIST rarely metastasizes to the lymph nodes (9,10). Under these circumstances, laparoscopic surgery is equivalent to traditional open surgery. Although in a retrospective study on dozens of cases, laparoscopic surgery for GIST and SMT was reported to be less invasive than open surgery, and the complications of both operations were equivalent (5,11–13). Moreover, it has been reported that laparoscopic resection of GIST <5 cm in diameter is as oncologically feasible as open surgery from medium- to long-term standpoints. The National Comprehensive Cancer Network Clinical Practice Guidelines and clinical practice guidelines for GIST in Japan recently suggested that experienced surgeons may consider the laparoscopic technique for tumors less than 5 cm in diameter (14,15). The stomach is a large organ centered in the abdomen, and in appropriate circumstances, the stomach can be partially resected with endoscopic stapling devices. As we previously reported, we aggressively apply laparoscopic resection to gastric GIST and achieve acceptable surgical results and oncologic outcomes (5).

SILS is a recent evolution in laparoscopic surgery that allows a number of forceps to be inserted via a single incision. The possible advantages of SILS include improved cosmesis and reduced tissue damage because fewer trans-abdominal ports are needed (16). In contrast, SILS has some disadvantages, most which are technical concerns: conflicts between the laparoscope and operating devices, in-line movement of instruments, limited

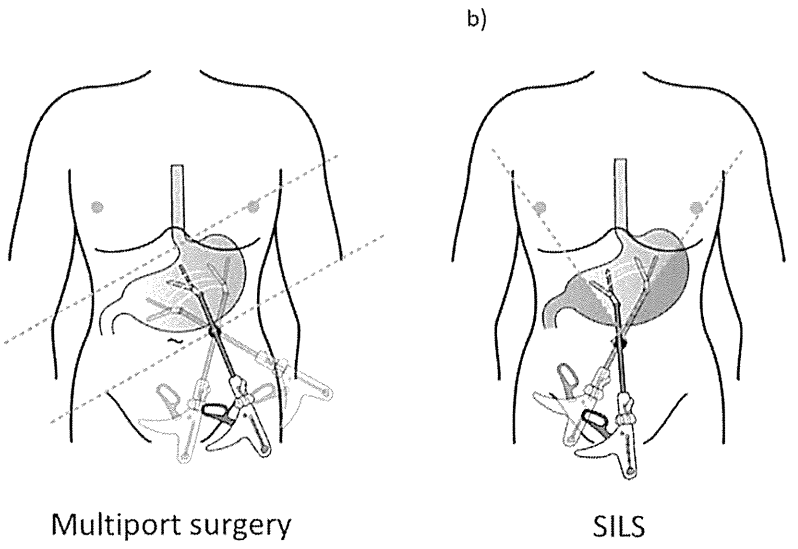

Figure 2 (a) In multiport laparoscopic surgery, a digital stapling device can be managed and is able to reach around the stomach. (b) However, in SILS, handling of a digital stapling device is circumscribed, and the device must be inserted in the direction of the long axis of the stomach.

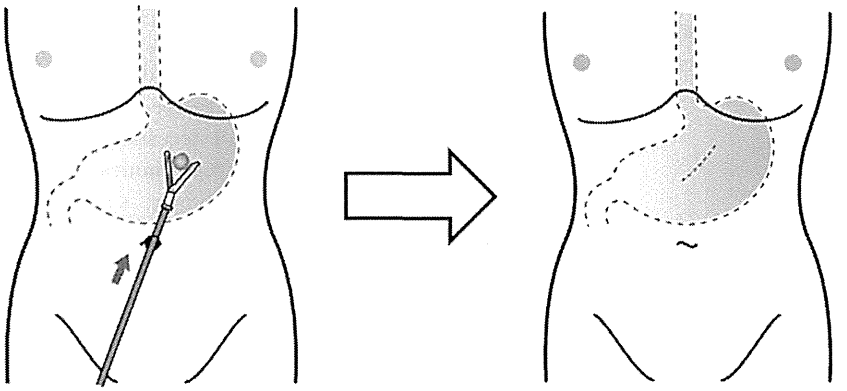

Figure 3 In SILS, the endoscopic stapling device can only be inserted in the direction of the long axis of the stomach.

organ retraction, and difficultly in tissue triangulation (17).

Although there are some reports that SILS has been safely performed for GIST (6,7,18,19), they did not deal with any technical issues (e.g. difficulty handling the endoscopic linear stapler) or how they were resolved. In the present series, the conflict among transumbilical devices was partially resolved by using a SILS access device, which allowed more flexible port placement. By ensuring that there was distance between each port, we could obtain a practical working angle between left-handed and right-handed instruments. As a result, most laparoscopic dissection could be completed with the conventional parallel technique. We also resolved the retraction issue by using a 2-mm loop retracting device. By carefully including the diaphragmatic fascia in the loop, we effectively retracted the left lateral segment of the liver. This retraction was robust and the surgical exposure was stabilized throughout the procedure.

One remaining challenge specific to single-incision laparoscopic partial gastrectomy, was transumbilical surgical stapling. In conventional multiport laparoscopic surgery, the stapling device is inserted via the left mid-abdomen. This allows for an adjustable staple line formation for virtually all lesions in the stomach (Figure 2). In SILS, the stapler is inserted via the umbilicus, so the insertion direction of the stapling device is almost always parallel to the organoaxial of the stomach (Figure 3). The stapling becomes further complicated as a result of the limited handling of the stapling device in the crowed transumbilical instruments. To accomplish appropriate gastric stapling in such adverse condition, we adopted the "move the ground" technique (Figure 4). Trying to adjust the staple line by moving the stapler is almost always unsuccessful. Instead of moving the stapler, we brought the lesion to the staple by using an articulated grasper. Although this technique requires prior wide mobilization of the stomach, it is extremely useful in SILS gastrectomy where handling of the stapling device is limited.

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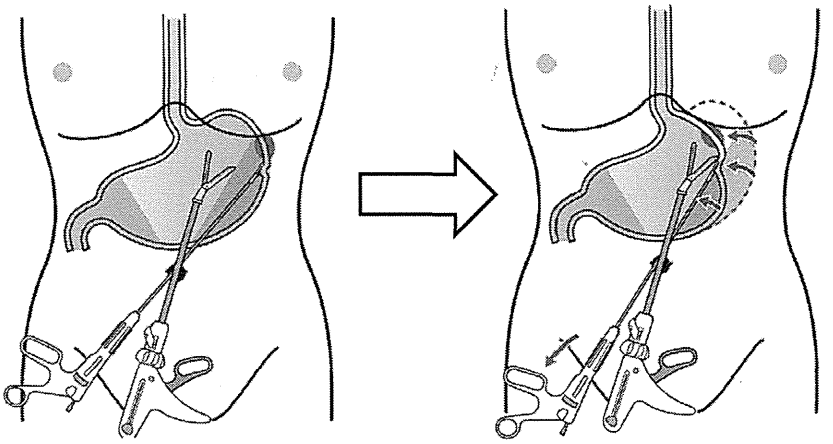

Figure 4 In a stapled resection, adjusting the stapling line by moving the stapler is almost always unsuccessful. Instead of moving the stapler, we brought the lesion to the stapler.

Any type of surgical approach for gastric SMT should be validated in terms of oncologic clearance and gastric remnant function. SILS is still a new technology, and therefore, we should carefully select candidates until we obtain conclusive data regarding oncologic and functional outcomes. At this time, we restrict the application of SILS to lesions on the anterior gastric wall or greater curvature that can be resected with a stapling device. Extended follow-up is mandatory to validate oncologic appropriateness for this small group of patients.

SILS is feasible, safe and reasonable for gastric SMT, without compromising transumbilical gastric stapling. This technique is an attractive and practical alternative to conventional multiport laparoscopy in carefully selected patients, and it offers improved cosmetic outcomes.

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