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Expression of vascular endothelial growth factor-C and vascular endothelial growth factor receptor-3 in esophageal squamous cell carcinoma

ITARU OMOTO, MASATAKA MATSUMOTO, HIROSHI OKUMURA, YASUTO UCHIKADO, TETSURO SETOYAMA, YOSHIAKI KITA, TETSUHIRO OWAKI, YUKO KIJIMA, HIROYUKI SHINCHI, SUMIYA ISHIGAMI, SHINICHI UENO and SHOJI NATSUGOE

Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medicine, Kagoshima University, Kagoshima 890-8520, Japan

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Abstract. Lymph node metastasis is one of the most important prognostic factors in esophageal squamous cell carcinoma (ESCC). Vascular endothelial growth factor (VEGF)-C and its receptor, VEGF receptor-3 (VEGFR-3), are key in the process of lymphangiogenesis. The present study immunohistochemically examined the expression of VEGF-C, VEGFR-3 and D2-40 in 119 patients with ESCC, and microlymphatic vessel density (MLVD) was calculated based on D2-40 expression counts. Positive expression of VEGF-C was found to correlate significantly with depth of tumor invasion, lymphatic invasion and lymph node metastasis ($P<0.001$, $P<0.0001$ and $P<0.0001$, respectively). Patients with deeper tumor invasion showed higher positivity of VEGFR-3 expression ($P<0.05$), while patients with lymph node metastasis showed higher MLVD ($P<0.05$). When patients were divided into three groups according to the expression of VEGF-C and VEGFR-3, patients with coexpression of VEGF-C and VEGFR-3 exhibited poorer prognosis and higher MLVD. The VEGF-C/VEGFR-3 axis is important in tumor lymphangiogenesis.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive types of gastrointestinal cancer, due to the relatively high risk of metastasis even in the early stage. In particular, lymph node metastasis is one of the most important prognostic factors (1). Tumor cells take advantage of the lymphatic vascular system to promote metastasis to the lymph nodes and beyond (2). Tumor-induced lymphangiogenesis promotes metastasis to regional lymph nodes and often represents the first step in tumor dissemination. Lymph node metastasis offers a major prognostic indicator for the progression of types of human cancer. Two members of the vascular endothelial growth factor (VEGF) family, VEGF-C and VEGF-D, reportedly induce not only angiogenesis, but also lymphangiogenesis via VEGF receptor (VEGFR)-2 and VEGFR-3 on lymphatic endothelial cells (3,4). These receptors not only regulate lymphangiogenesis, but also enhance lymphatic metastasis (5). In addition, VEGF-C and VEGFR-3, which together have been proposed as a marker for lymphatic endothelial cells, have recently been reported to be expressed by tumor cells in correlation with the invasion, metastasis and progression of cancer cells (6-8).

Several studies have previously examined the roles of the VEGF-C/VEGFR-3 axis and lymphangiogenesis. Lymphangiogenesis is a key factor in nodal metastasis and a prognostic factor for various carcinomas of the esophagus (9), stomach (10-12), colorectum (13), lung (14), cervix (15,16) and prostate (17,18).

The present study aimed to clarify whether expression of VEGF-C and VEGFR-3 in the tumor cells of ESCC correlates with tumor lymphangiogenesis, lymph node metastasis and other clinicopathological factors. In addition, it was examined whether VEGF-C and VEGFR-3 have potential as targets of molecular therapies.

Materials and methods

Patients. In total, 119 patients with ESCC (108 males and 11 females) who underwent curative esophagectomy with lymph node dissection between 1996 and 2003 at the Kagoshima University Hospital (Kagoshima, Japan) were enrolled. Patient ages ranged between 38 and 86 years (mean, 65.3 years). Transthoracic esophagectomy by right and left thoracotomy was performed in 89 (74.8%) and six patients (4.2%), respectively. In addition, transhiatal esophagectomy without thoracotomy was performed in 21 patients (17.6%)

Correspondence to: Dr Itaru Omoto, Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan
E-mail: itaru@m3.kufm.kagoshima-u.ac.jp

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and abdominal lower esophagectomy was performed in three patients (3.4%). Three-field lymphadenectomy (cervical, mediastinal and abdominal regions) was performed in 42 patients (35.3%), two-field lymphadenectomy (mediastinal and abdominal regions) in 74 patients (62.2%) and one-field (abdominal region) lymphadenectomy in the remaining three patients. The median number of removed lymph nodes was 42 (range, 5-136) and the number of patients with R0 and R1 resection was 107 and 12, respectively. None of these patients underwent endoscopic mucosal or palliative resection, preoperative chemotherapy or radiotherapy, or exhibited synchronous or metachronous cancer in other organs. Specimens of cancer and non-cancerous adjustment tissues were collected from the patients after informed written consent had been obtained in accordance with the institutional guidelines of the hospital.

Clinicopathological observations were based on the criteria of the TNM classification for esophageal carcinoma of the International Union Against Cancer (19). In total, 29 of the ESCCs were classified as well-differentiated, 68 as moderately differentiated and 22 as poorly differentiated. In addition, 26 of the tumors were located in the upper third of the esophagus, 60 in the middle third and 33 in the lower third. Overall, 40 patients exhibited pT1 tumors, 18 exhibited pT2 tumors and 61 exhibited pT3 tumors. Lymph node metastasis was found in 76 of the 119 patients (63.9%) and lymphatic and venous invasion was identified in 74.8% (89/119) and 66.4% (79/119) of patients, respectively. All the M1 tumors exhibited distant lymph node metastases. Each patient was followed up after discharge with a chest X-ray every 1 to 3 months, computed tomography every 3 to 6 months and ultrasonography every 6 months. Bronchoscopy and endoscopy were performed when necessary. Postoperative follow-up data were available for all patients with a median follow-up period of 39 months (range, 1-137 months). Consequently, 51 patients exhibited relapsed disease in the follow-up period.

Immunohistochemistry. Once the primary lesions had been fixed in 10% formaldehyde and routinely embedded in paraffin, 3-μm-thick sections were prepared for immunohistochemistry. Sections were deparaffinized in xylene, rehydrated in graded ethanol and incubated in 0.3% H_2O_2 solution in methanol for 30 min to block endogenous peroxidases. All sections were autoclaved in 10 mM sodium citrate (pH 6.0) for 10 min and allowed to cool at room temperature. Following washing three times with phosphate-buffered saline for 5 min each, sections were treated with 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) for 30 min at room temperature.

Sections were incubated overnight at 4°C with the following three antibodies: Mouse anti-VEGF-C monoclonal (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-VEGFR-3 polyclonal (1:200; R&D Systems, Wiesbaden, Germany) and mouse anti-D2-40 monoclonal (1:50; Dako, Carpinteria, CA, USA). These reactions were developed using an avidin-biotin immunoperoxidase technique (ABC method). The reaction was visualized using the Vectastain Elite ABC kit and 3,3'-diaminobenzidine solution (Vector Laboratories, Burlingame, CA, USA). Sections were then slightly counterstained with hematoxylin.

Expression of VEGF-C and VEGFR-3 in >30% of the cells examined was considered to represent a positive result (9). Expression of VEGF-C and VEGFR-3 was evaluated in 10 fields of ≥100 cells each using high-power (magnification, x200) light microscopy (BX50, Olympus, Tokyo, Japan). All immunostained slides were evaluated by two independent observers (I.O. and M.M.).

Evaluation of microlymphatic vessel density (MLVD). Vessel count was assessed by light microscopy in areas of tumor containing the highest numbers of capillaries at the invasive edge. Highly vascular areas were identified by scanning tumor sections at low power (magnification, x40 and x100; DP71, Olympus). In total, six areas showing the highest degree of neovascularization were identified, vessel count was performed in a x200 field (x20 objective and x10 ocular) and the mean count for the six fields was determined as MLVD. As previously described by Weidner *et al*, identification of a vessel lumen was not necessary for a structure to be defined as a vessel (20).

Statistical analysis. Statistical analysis was performed using JMP® 5.0.1 (SAS Institute Inc., Cary, NC, USA), Student's t-test, χ^2 test, Kaplan-Meier method and log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of VEGF-C, VEGFR-3 and D2-40 in esophageal carcinoma tissue. Expression of VEGF-C (Fig. 1A) and VEGFR-3 (Fig. 1B) was distributed throughout the cytoplasm of cancer cells. Rates of positive VEGF-C and VEGFR-3 expression were 42.9% (51/119) and 28.6% (34/119), respectively. D2-40 expression was detected in lymphatic endothelial cells (Fig. 1C) and the mean MLVD was 25.8±13.4/field (range, 0-68/field).

Correlation between clinicopathological factors and expression of VEGF-C and VEGFR-3. Table I shows the correlation between VEGF-C expression and pathological observations. VEGF-C expression was found to correlate significantly with tumor depth, presence of lymph node metastasis and lymphatic invasion ($P<0.0001$ each). Table I also shows the correlation between VEGFR-3 expression and pathological observations. VEGFR-3 expression was found to correlate significantly with tumor depth and lymphatic invasion ($P=0.01$ and $P=0.032$, respectively). Although, the incidence of lymph node metastasis tended to occur in patients with positive expression of VEGFR-3; however, the correlation was not significant.

Correlation between MLVD and expression of VEGF-C and VEGFR-3. Correlations between the expression of VEGF-C and VEGFR-3 and MLVD are shown in Figs. 2A and B. VEGF-C and VEGFR-3 expression was found to correlate significantly with high MLVD ($P=0.0033$ and $P=0.014$, respectively). Mean MLVD was 29.95±14.12/field in the VEGF-C-positive group, 22.73±12.03 in the VEGF-C-negative group, 30.55±15.63/field in the VEGFR-3-positive group and 23.94±11.98 in the VEGFR-3-negative group.

Correlation between prognosis and expression of VEGF-C and VEGFR-3. Five-year survival rates were analyzed according to the expression of VEGF-C and VEGFR-3. The 5-year survival rate was significantly higher in VEGF-C-negative patients

Table I. Correlation between VEGF-C and VEGFR-3 expression and clinicopathological factors in 119 ESCC patients.

Factors	VEGF-C-positive expression (n=51), n (%)	P-value	VEGFR-3-positive expression (n=34), n (%)	P-value
Histopathological grading		0.4954		0.0859
Grade 1-2 (n=97)	43 (44)		31 (32)	
Grade 3 (n=22)	8 (36)		3 (14)	
Depth of tumor invasion		<0.0001		0.0140
T1 (n=40)	7 (18)		5 (13)	
T2 (n=18)	6 (33)		5 (28)	
T3 (n=61)	38 (62)		24 (39)	
Lymphatic invasion		<0.0001		0.0327
Negative (n=30)	2 (6)		5 (16)	
Positive (n=89)	49 (55)		30 (33)	
Lymph node metastasis		<0.0001		0.3343
Negative (n=43)	6 (14)		10 (23)	
Positive (n=76)	45 (58)		24 (32)	

VEGF-C, vascular endothelial growth factor-C; VEGFR-3, vascular endothelial growth factor receptor-3; ESCC, esophageal squamous cell carcinoma.

Table II. Uni- and multivariate analyses of prognostic factors.

Factors	Univariate P-value	Multivariate P-value	95% confidence interval	Hazard ratio
pT1b/pT2-3	<0.0001	0.0017	1.188-2.256	1.610
$pN^{-/+}$	0.0002	0.0095	1.095-2.031	1.473
$VEGF\text{-}C^{-/+}$	0.0005	0.1567	0.919-1.649	1.237
$VEGF\text{-}C^{+}$, $VEGFR\text{-}3^{+}$ and other patterns	0.0210	0.7295	0.760-1.498	0.061

VEGF-C, vascular endothelial growth factor-C; VEGFR-3, vascular endothelial growth factor receptor-3.

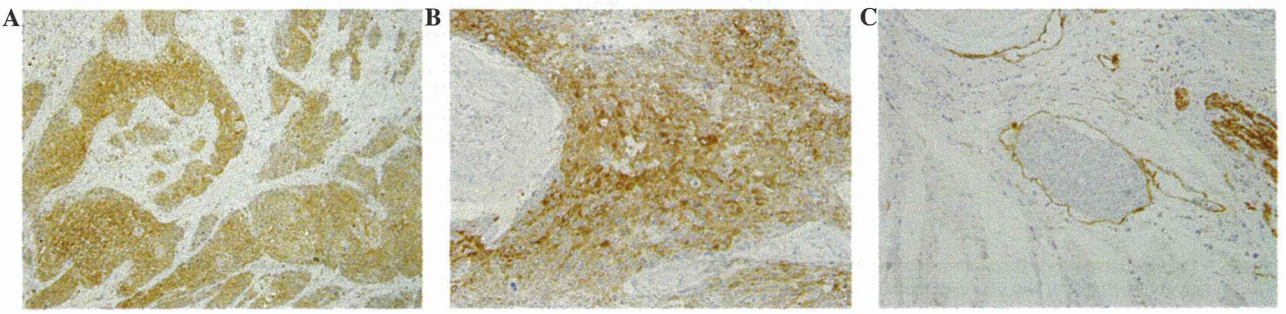

Figure 1. Expression of VEGF-C, VEGFR-3 and D2-40 in esophageal squamous cell carcinoma tissue. (A) VEGF-C (magnification, x100) and (B) VEGFR-3 (magnification, x200) were distributed throughout the cytoplasm of cancer cells. (C) D2-40 expression was detected in lymphatic endothelial cells (magnification, x200). VEGF-C, vascular endothelial growth factor-C; VEGFR-3, vascular endothelial growth factor receptor-3.

(55%) than in patients with positive expression (31%; P=0.0006; Fig. 3A). No significant difference in 5-year survival rate was found according to the expression of VEGFR-3 (Fig. 3B).

Prognosis according to the expression of VEGF-C and VEGFR-3. The 5-year survival rate was significantly higher in the double-negative group than in the double-positive group (P=0.0032; Fig. 3C).

Uni- and multivariate analyses of survival. Univariate analysis showed that the following factors were significantly associated with postoperative survival: Tumor depth, lymph

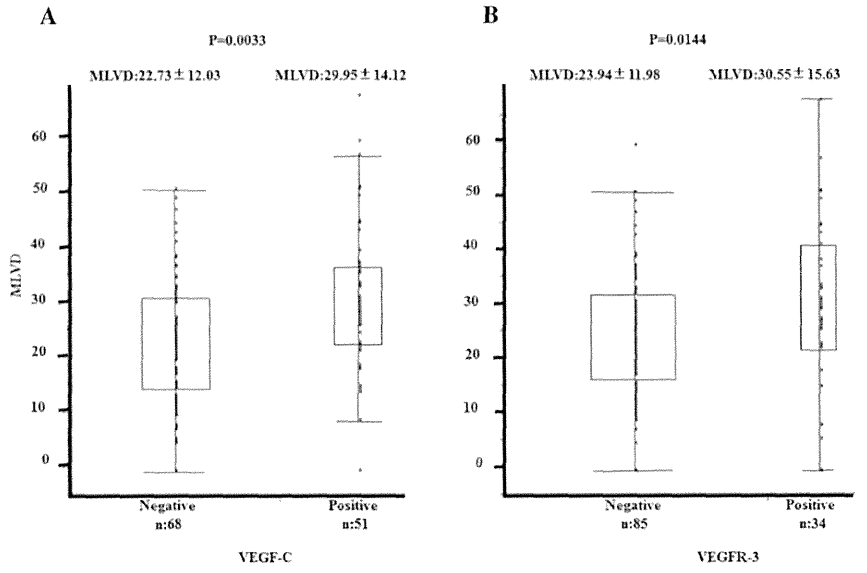

Figure 2. Correlation between MLVD and expression of (A) VEGF-C and (B) VEGFR-3 in esophageal squamous cell carcinoma. MLVD, microlymphatic vessel density; VEGF-C, vascular endothelial growth factor-C; VEGFR-3, vascular endothelial growth factor receptor-3.

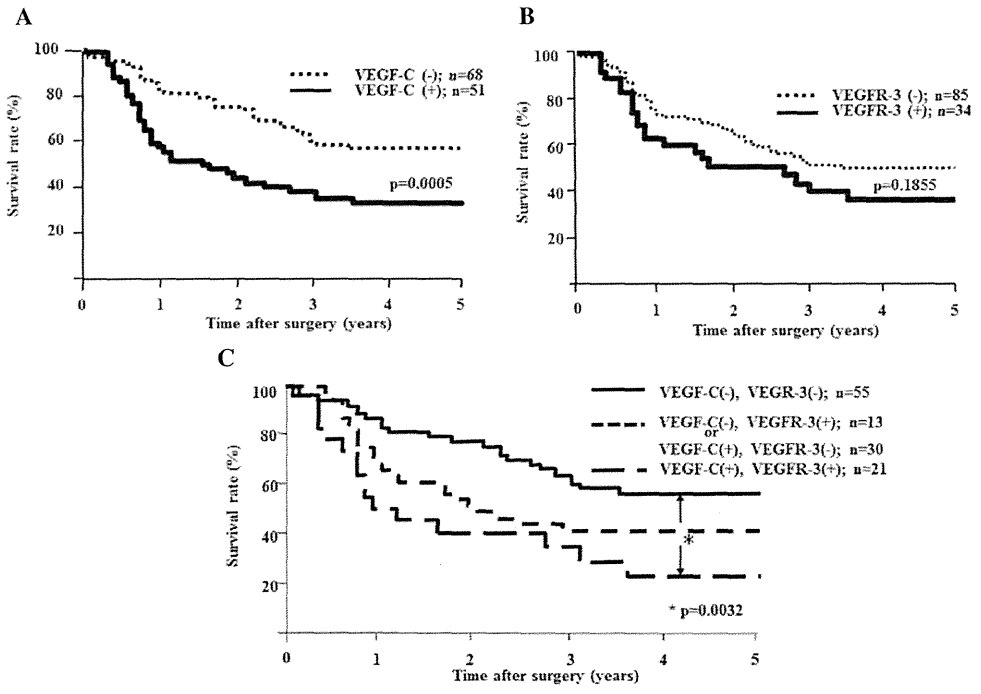

Figure 3. Postoperative survival curves according to (A) VEGF-C, (B) VEGFR-3 and (C) VEGF-C and VEGFR-3 expression. VEGF-C, vascular endothelial growth factor-C; VEGFR-3, vascular endothelial growth factor receptor-3.

node metastasis, VEGF-C expression, and coexpression of VEGF-C and VEGFR-3 ($P<0.05$). Multivariate regression analysis indicated depth of tumor invasion and lymph node metastasis as independent prognostic factors (Table II).

Discussion

Lymphangiogenesis represents an important step in tumor progression and metastasis. Previous studies have revealed that tumors actively induce their own networks of lymphatics that connect with surrounding lymphatic vessels (21-25). The transport of tumor cells by lymphatic vessels represents the most common pathway for initial dissemination, with cancer spread by afferent lymphatics following routes of natural drainage (26-29). Previously, two members of the VEGF family, VEGF-C and VEGF-D, have been associated with lymphangiogenesis and are known as natural ligands for VEGFR-3 (30,31). The present study focused on the expression

of VEGF-C and VEGFR-3 and MLVD in ESCC, and evaluated the involvement of the VEGF-C/VEGFR-3 signaling pathway on lymphangiogenesis in ESCC.

In the present study, D2-40 antibody, which reacts with an oncofetal antigen present in fetal germ cells, is a highly reliable lymphatic endothelial marker (32), was first used to detect microlymphatic vessels. Numerous studies have previously indicated that the immunostaining for D2-40 allows specific evaluation of lymphatic invasion and MLVD in types of human cancer (10,33). In the present study, D2-40-expressing microvessels were found in carcinoma tissues, particularly ESCC with lymph node metastases.

With regard to the correlations with clinicopathological features, VEGF-C expression was found to correlate well with several factors, including tumor depth, lymphatic invasion, lymph node metastasis and MLVD, while close correlations with VEGFR-3 expression were limited to tumor depth and MLVD. This may suggest the existence of other pathways for lymphatic spread, but the two molecules were found to closely correlate with each other. These observations suggested that VEGF-C is the most important factor in lymphatic spread and that overexpression of VEGF-C and VEGFR-3 facilitates tumor lymphangiogenesis, resulting in the proliferation of lymphatic vessels. In other words, VEGF-C induces tumor lymphangiogenesis by stimulating VEGFR-3 expression on lymphatic endothelial cells.

Next, the prognosis of ESCC patients was analyzed and patients with overexpression of VEGF-C showed poorer outcomes than those without overexpression, while VEGFR-3 expression was not found to correlate significantly with survival rate. However, expression of VEGF-C and VEGFR-3 resulted in poorer outcomes than other combinations. These results suggested that VEGFR-3 expression in ESCC may have effects only in the presence of sufficient VEGF-C. As previously described in several reports, the VEGF-C/VEGFR-3 axis is critical in cancer progression by inducing lymphangiogenesis and facilitating the mobility of several types of cancer cells. The results of the present study support these previous observations with regard to the role of the VEGF-C/VEGFR-3 axis in the induction of lymphangiogenesis that results in the lymphatic spread of ESCC. MLVD was found to significantly correlate with the VEGF-C/VEGFR-3 system and may present a risk factor for lymph node metastasis and a prognostic factor in ESCC.

Previously, various anti-angiogenic treatments have been applied in clinical situations. VEGF-A and VEGFR-2 are currently the main focus of study. Bevacizumab is a humanized monoclonal antibody against VEGF-A and aflibercept (VEGF-Trap) is a soluble fusion protein for the extracellular domain of VEGFR-1 and VEGFR-2 and the Fc region of immunoglobulin G. These agents neutralize VEGF-A, preventing tumor angiogenesis. VEGFR tyrosine kinase inhibitors, such as sunitinib and sorafenib, are also effective in anti-angiogenic tumor therapy by inhibiting VEGFR signaling. Anti-VEGF drugs currently appear promising as therapies for various cancer patients.

Conversely, lymphangiogenesis shows similar biological mechanisms to angiogenesis. VEGF-C and VEGFR-3 expression, as well as MLVD, may serve as prognostic biomarkers in patients with ESCC (34). Lymphangiogenesis is activated in cancer and inflammation, but is largely inactive in normal physiology, suggesting the therapeutic potential of targeting the underlying mechanisms. As demonstrated in the results of the current study, VEGF-C and VEGFR-3 signaling appear essential for the development of lymphatic vessels and, thus, provide a promising target for the inhibition of tumor lymphangiogenesis. Previously, Burton *et al* (35) emphasized the importance of inhibiting prostate cancer by blockade of the VEGF-C/VEGFR-3 axis. The authors used a VEGF-C ligand trap and antibody directly against VEGFR-3, which significantly reduced tumor lymphangiogenesis and metastasis to regional lymph nodes and distal vital organs without influencing tumor growth.

An additional potential application to clinical situations is the early detection of cancer spread. Previously, Mumprecht *et al* (36) applied immune-positron emission tomography with a lymphatic-specific antibody, LYVE-1, to detect metastases in the early stage. The resulting images suggested the usefulness of this approach in determining the progression of diseases with a marked lymphangiogenic component. In the present study, overexpression of VEGF-C and VEGFR-3 was suggested to induce lymphatic proliferation of the tumor. Obtaining information predictive of lymphatic spread and lymph node metastases must be useful for selecting appropriate strategies for ESCC treatment.

The VEGF-C/VEGFR-3 axis is important in tumor lymphangiogenesis. Targeting the VEGF-C/VEGFR-3 axis may be therapeutically important for cancer metastasis (28,37). The results of the present study may be beneficial for the treatment of patients with ESCC, and new drugs aimed at blocking the VEGF-C/VEGFR-3 axis may be useful for limiting lymph node metastasis. However, several issues remain with regard to the frequency, mechanisms and biological importance of lymphatic metastases. Numerous growth factors appear to be important in determining the lymph node metastatic potential of ESCC. Future study is necessary to clarify the molecular pathways and introduce novel therapeutic options.

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Immunohistochemical Evidence of Association Between Ghrelin Expression and Tumor Growth in Esophageal Carcinoma

ITARU OMOTO, MASATAKA MATSUMOTO, YASUTO UCHIKADO, YOSHIAKI KITA, TOSHIHIDE SAKURAI, KEN SASAKI, TETSURO SETOYAMA, HIROSHI OKUMURA, TETSUHIRO OWAKI, SUMIYA ISHIGAMI and SHOJI NATSUGOE

Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, Sakuragaoka, Kagoshima, Japan

Abstract. Background: Ghrelin, an orexigenic peptide, is primarily produced and secreted by the gastrointestinal tract. As far as we are aware of, there is no evidence of ghrelin expression in esophageal squamous cell carcinoma (ESCC). Materials and Methods: Two hundred and ten patients with ESCC who underwent surgical resection were enrolled in this study. We immunohistochemically investigated ghrelin expression in primary ESCC specimens and analyzed the relationship with clinicopathological factors. Results: High ghrelin expression was observed in 61 patients (29.0%). Depth of tumor invasion and histological differentiation were statistically associated with ghrelin expression. As for depth of tumor invasion, the deeper it was, the higher was the expression of ghrelin. Well-differentiated tumors had a significantly higher proportion of ghrelin-expressing cells than other types. Conclusion: Ghrelin expression correlated with tumor depth and tumor differentiation, suggesting an important role of ghrelin in tumor growth in ESCC.

Malignant tumors are characterized by extensive invasion, metastasis, and marked cachexia. Several reports have referred to the role of ghrelin which stimulates the release of growth hormone (GH), in malignant cell invasion and proliferation. Murata *et al.* first suggested the possibility of proliferative role of ghrelin in a hepatoma cell line (1). Ghrelin is a 28-amino acid peptide hormone, produced mainly in the stomach, by neuroendocrine cells (X/A-like cells in rodents and P/D1 cells in humans) in the fundus, and secreted into the circulation (2-4). Since its discovery, it has been implicated in a wide range of physiological activities, including the control of food intake and metabolism (5-8). Obese people have been shown to have low fasting ghrelin levels compared to those with normal body-mass index (BMI) in an examination to limit uptake of fat (5, 6, 9, 10). In contrast, cachectic patients exhibit higher fasting ghrelin levels (5, 6, 11, 12).

Ghrelin is produced mainly in a non-acylated form. Orexigenic and growth hormone-stimulating effects of ghrelin depend on its acylation with an octanoyl fatty acid residue at the serine-3 position (4, 6). Lower levels of ghrelin are found in the small and large intestine, cells of the pancreatic islet, kidney, testis, placenta, and immune cells (13-19). Locally produced ghrelin possibly affects cells with which can act as receptors for ghrelin. Receptors of ghrelin are known as growth hormone secretagogue receptors (GHS-Rs) (20-23). GHS-Rs have two sub-types: type Ia (GHS-RIa) transduces the GH-releasing effect of GHS, whereas type 1b GHS-RIb is a non-spliced, non-functional receptor mRNA variant. GHS-RIa is mainly expressed in the pituitary, hypothalamus and at low levels in other brain regions such as ventral tegmental area, *substantia nigra*, *nucleus tractus solitaries*, and *hippocampus* as well as peripheral tissue such as the heart, lungs, pancreas, intestine, kidneys, and adipose tissue (19, 24-27).

Some groups have reported the proliferative effect of ghrelin in neuronal, adrenal, prostatic, adipose, mammary, chondroblastic and pancreatic cells (7, 20, 21, 28-34). In contrast there are reports of the anti-proliferative role of ghrelin in few cells (35). Furthermore, some authors reported that ghrelin expression is suppressed in malignant tumors (36, 37), while others emphasized on ghrelin expression in tumors (38, 39). Recently, much effort has been put into research regarding the role of ghrelin in cancer, which is still unclear.

Correspondence to: Itaru Omoto, MD, Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima, 890-8520, Japan. Tel: +81-99-275-5361, Fax: +81-99-265-7426, e-mail: itaru@m3.kufm.kagoshima-u.ac.jp

Key Words: Ghrelin, esophageal cancer, tumor progression.

Esophageal cancer is the most aggressive type of cancer among gastrointestinal cancers because of the existence of metastases even in the early stages (40). Mottershead *et al.* reported the negligible expression of ghrelin in esophageal adenocarcinoma (37). Nevertheless, as far as we are aware of, there have been no reports on the expression of ghrelin in esophageal squamous cell carcinoma (ESCC). Herein, we examined ghrelin expression in resected specimens of ESCC and its relationship with clinicopathological features.

Materials and Methods

Patients. We enrolled two hundred and ten patients with ESCC (189 males and 21 females) who underwent curative esophagectomy with lymph node dissection between 1990 and 2004 at the Kagoshima University Hospital, Kagoshima, Japan (Table I). The age of the patients ranged from 38 to 86 years (mean=64.8±9.3 years). None of them underwent endoscopic mucosal resection, palliative resection, preoperative chemotherapy, or radiotherapy. Patients with synchronous or metachronous multiple cancers in other organs were excluded. Specimens of cancer tissues and noncancerous adjacent tissues were collected from the patients after informed consent had been obtained in accordance with the institutional guidelines of our hospital.

Clinicopathological findings were based on the criteria of the TNM classification for esophageal carcinoma of the International Union Against Cancer (41). We classified 67 of the ESCCs as well differentiated, 106 as moderately-differentiated, and 37 as poorly-differentiated. Forty of the tumors were located in the upper third of the esophagus, 102 in the middle third, and 68 in the lower third. Regarding tumor depth, 63 patients had pT1 tumors, 32 had pT2 tumors and 115 had pT3 tumors. Lymph node metastasis was found in 138 of the 210 patients (65.7%). Lymphatic and venous invasion were found in 74.3% (156/210) and 56.7% (119/210) of the patients, respectively (Table I).

Immunohistochemistry. After primary lesions were fixed in 10% formaldehyde and routinely embedded in paraffin, 3-μm thick sections were prepared for immunohistochemistry. Sections were de-paraffinized in xylene, rehydrated in graded ethanol, and incubated in 0.3% H_2O_2 solution in methanol for 30 min to block endogeneous peroxidases. All sections were autoclaved in 10 mM sodium citrate (pH 6.0) for 10 min and allowed to cool at room temperature. After washing three times with Phosphate buffered saline (PBS) for 5 min each, the sections were treated with 1% bovine serum albumin for 30 min at room temperature. The sections were incubated overnight at 4°C with the mouse monoclonal antibody to human ghrelin (1: 200; Abcam, Tokyo, Japan). These reactions were developed with an avidin-biotin immunoperoxidase technique (ABC method). The reaction was visualized using the Vectastain Elite ABC kit and a 3,3'diaminobenzidine solution (Vector Laboratories, Inc., Burlingame, CA, USA). Sections were then slightly counterstained with hematoxylin. Normal gastric mucosa of corpus was utilized as a positive control.

The expression of ghrelin was evaluated in 10 fields each containing 100 tumor cells using high-power microscopy (×200) expressing the proportion of positively stained cells as a percentage of total cells examined. All immunostained slides were evaluated by two independent observers (IO and MM). According to the proportion of ghrelin expression, patients were divided into two groups (≥20%: high expression, <20%: low expression), because the mean proportion of ghrelin-expressing cells in the patients' tumor was 17.19±16.66%.

Table I. *Clinicopathological charercteristics of ESCC patients.*

Factor	N (%)
Gender	
Male	189 (90.0)
Female	21 (10.0)
Age	64.9±9.3
Tumor location	
Upper	40 (19.0)
Middle	102 (48.6)
Lower	68 (32.4)
Differentiation	
Grade 1	67 (31.9)
Grade 2	106 (50.5)
Grade 3	37 (17.6)
Depath of tumor invasion	
pT1	63 (30.0)
pT2	32 (15.2)
pT3	115 (54.8)
Lymph Node Metastasis	
Absent	72 (34.3)
Present	138 (65.7)

Statistical analysis. Statistical analysis consisting of Student's *t*-test, the Chi-square test, the Kaplan-Meier method, and the log-rank test was performed using the JMP IN version 5.0.1 software system (SAS institute Inc., Cary, NC, USA). A *p*-value of less than 0.05 was considered to indicate statistical significance.

Results

Expression of ghrelin in esophageal carcinoma tissue. The expression of ghrelin was expressed throughout the cytoplasm of cancer cells, but was not found in the nucleus of cancer cells (Figure 1). Sixty-one patients had high expression of ghrelin. Ghrelin was expressed diffusely throughout the tumor without heterogenity. No expression was observed in intratumoral stromal or inflammatory cells.

Correlation between clinicopathological factors and expression of ghrelin. Table II shows the correlation between ghrelin expression and pathological findings. High ghrelin expression was significantly correlated with depth of tumor invasion, pathological stage, tumor differentiation and venous invasion ($p<0.0001$, $p<0.005$, $p<0.0005$ and $p<0.05$, respectively). As for the proportion of lymph node metastasis and lymphatic invasion, patients with high expression of ghrelin had a higher incidence than those with lower expression, although the difference was not significant ($p=0.21$ *vs.* 0.10). Tumor location and recurrence rate were

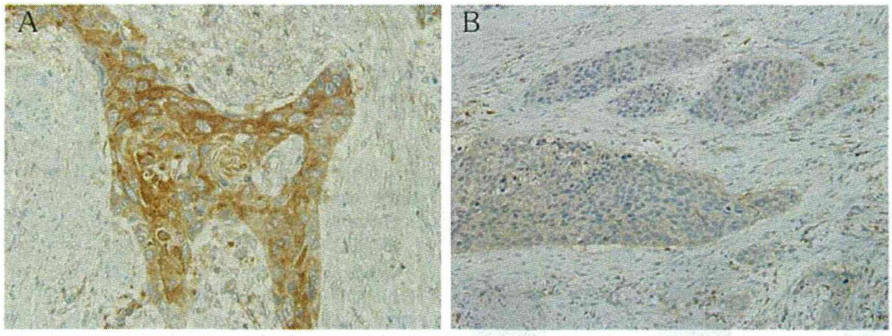

Figure 1. *Expression of ghrelin in esophageal carcinoma tissue. Ghrelin was distributed throughout the cytoplasm of cancer cells. A: High expression (×400); B: low expression (×200).*

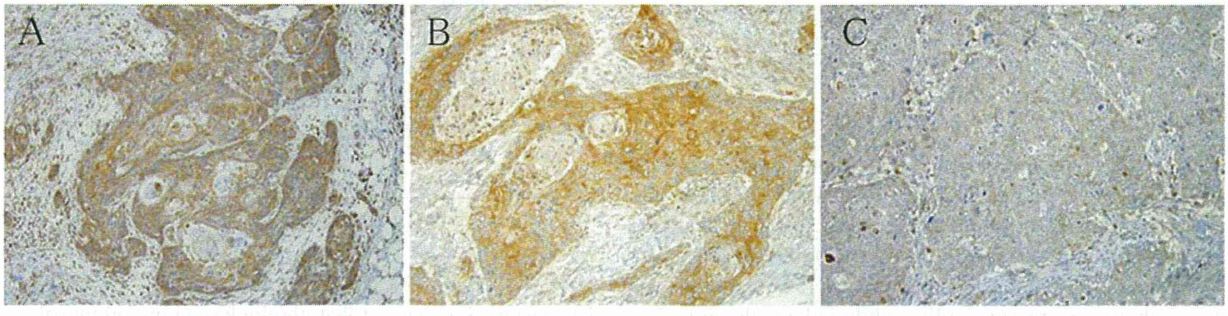

Figure 2. *Immunohistochemical expression of ghrelin in esophageal squamous cell carcinoma according to tumor differentiation. Well differentiated tumor has high density, while poorly differentiated tumor (C) has low density; B: moderately differentiated tumor (×200).*

not statistically correlated with the expression of ghrelin. The group of patients with tumors of advanced clinical stage had a higher proportion of ghrelin-expressing cells. These results suggest that in ESCC, cancer cells express ghrelin and it is possible that its expression has a relationship with tumor growth.

As for the depth of tumor invasion, the deeper it was, the higher the proportion of ghrelin-expressing cells was. However, well-differentiated tumors had a higher proportion of ghrelin expressing cells than other types (Table II). We did not evaluate the density of staining in detail, however, almost all sections, well- and moderately-differentiated tumors had a high density, and poorly-differentiated tumors a low density (Figure 2). We compared the proportion of ghrelin-expressing cells between tumors of different differentiation, and well-differentiated tumors had a significantly higher proportion of expression than the others (p=0.0026, Figure 3). These results suggest that tumor cells, particularly in well-differentiated tumors, endogenously recruit ghrelin to promote their growth, however, ghrelin does not appear to promote their differentiation.

Correlation between prognosis and expression of ghrelin. The five-year survival rate was analyzed according to the expression of ghrelin. No significant differences were found in the expression of ghrelin (Figure 4). According to Figure 4, in the short term, the difference in the one-year survival rate between the high and low expression groups. Approached borderline statistical significance (p=0.072, log rank test); hence there is a possibility that ghrelin expression in ESCC is of relevance regarding short-term survival.

Discussion

As far as we are aware of this is the first study to show a correlation between ghrelin expression and clinicopathological findings in ESCC. This study revealed the existence of ghrelin-expressing cells in ESCC.

Previous studies have shown that immunoreactivity for ghrelin was found in gastric endocrine tumors, pancreatic endocrine tumors, intestine endocrine tumors, lung carcinoids (42), prostate cancer (33, 43, 44), tesiticular

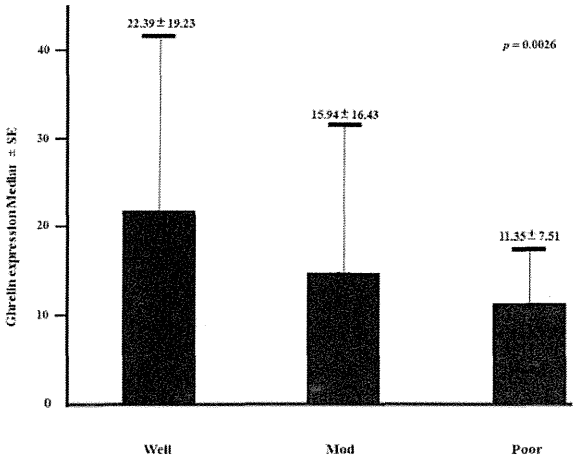

Figure 3. *Proportion of ghrelin-expressing cells by tumor differentiation. The tumors with lower differentiation had a higher proportion of ghrelin-expressing cells (p=0.0026).*

Table II. *Correlation between positive expression rate of Ghrelin and clinicopathological factors.*

Factor	Ghrelin high expression group n=61 (%)	Ghrelin low expression group n=149 (%)	*p*-Value
Diffferentiation			0.0002
Grade 1 (n=67)	31 (46)	36 (54)	
Grade 2 (n=106)	26 (25)	80 (75)	
Grade 3 (n=37)	4 (11)	33 (89)	
Depath of tumor invasion			<0.0001
T1 (n=63)	7 (11)	56 (89)	
T2 (n=32)	7 (22)	25 (78)	
T3 (n=115)	47 (41)	68 (59)	
pStage			0.0039
pStage 1/2 (n=105)	21 (20)	84 (80)	
pStage 3/4 (n=105)	40 (38)	65 (62)	
Vessel invasion			0.0226
Negative (n=91)	19 (21)	72 (79)	
Positive (n=119)	42 (35)	77 (65)	
Lymphatic invasion			0.1032
Negative (n=54)	11 (20)	43 (80)	
Positive (n=156)	50 (32)	106 (68)	
Lymph node matastasis			0.2100
Negative (n=72)	17 (24)	55 (76)	
Positive (n=138)	44 (32)	94 (68)	

tumors (45), gastrointesitinal carcinoids (46), pituitary adenomas (47), gastric adenocarcinoma (36, 37), salivary gland tumors (36), colorectal cancer (48), esophageal adenocarcinomas (37), breast cancers (31) (49), renal cancers (50), and oral squamous cell carcinomas (51). Some tumors have ghrelin-expressing cells, while others do not. The discrepancies among the results may be related to tissue differences, suggesting that the different genetic and epigenetic backgrounds of tissues as well as their embryological origins might contribute to differences in ghrelin expression in different cancer types.

Our results showed that ghrelin expression in ESCC was significantly correlated with depth of tumor invasion, pathological stage, tumor differentiation and venous invasion. As for tumor differentiation, the results were compatible with those of Rindi *et al.* (42) and Waseem *et al.* (48). They reported that ghrelin expression decreases with poorer differentiation. Rindi *et al.* suggested that the presence of protoendocrine cells void of hormonal storage was the main reason for the absence of ghrelin in poorly-differentiated endocrine tumors (42). Waseem *et al.* suggested that ghrelin was not required for enhanced malignant cell behavior in colorectal malignancy, (48) and also suggested that the ghrelin system contributes to the spread of malignancy rather than increasing the grade of the tumor as many other growth factors do, including insulin and insulin like growth factors (52). Our results also suggest that well-differentiated tumors of ESCC with high expression of ghrelin may have deeper invasion and be of more advanced stage. Furthermore, as we have indicated, ghrelin has a proliferative effect on malignant cells (1, 7, 20, 21, 28-34). Our results also suggest that ghrelin has an important role in carcinogenesis and cancer promotion.

Regarding the possibility of ghrelin being used as a tumor marker, in tumors expression ghrelin, Gronberg *et al.* examined breast cancer samples immunohistochemically and suggested that ghrelin expression correlated with lower risk for recurrence and cancer-related death (49). In contrast, another report by Jeffery *et al.* revealed that ghrelin treatment significantly increased the proliferation of cancer cells, and that low grade-carcinomas strongly expressed ghrelin and its receptor. They suggested that members of the ghrelin signaling axis may become novel markers for breast cancer and potential therapeutic targets (31). Alnema *et al.* also immunohistochemically investigated oral squamous cell carcinoma, and suggest that ghrelin expression in cancer was lower than in benign tissue, and so it might be helpful to distinguish malignant from benign tumors (51). Our results suggest that if we examine ghrelin expression in ESCC biopsy tissues, it may be useful in making a diagnosis of depth and differentiation. In this study, the five-year survival rate had no statistical significance, but there was a trend that the one-year survival rate for those with positive ghrelin expression was worse than for those without expression. Thus, it is still unclear whether ghrelin expression is truly a marker of malignancy.

Murphy *et al.* reported that lower serum level of ghrelin was associated with an increase of the risk of ESCC (53).

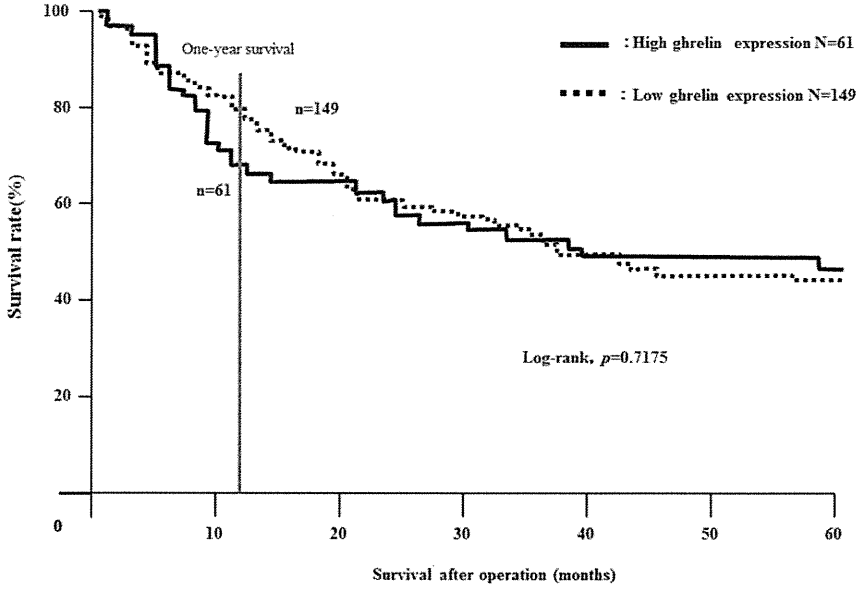

Figure 4. *Postoperative survival curves according to expression of ghrelin. There was no significant difference between tumors with high and low expression (p=0.7175, log-rank test).*

Waseem *et al.* examined correlation between serum ghrelin level and clinicopathological factors of patients with colorectal cancer, resulting in close correlation only with BMI change over six months (48). Other investigators also have tried to find if there is any relationship between serum ghrelin levels and malignancies, however, none have yet reported such correlation (54-59). The relationship between serum ghrelin levels and ghrelin expression in tumors remains unclear.

In treatment of cancer cachexia, several trials have been carried-out on ghrelin administration. The rodent model of cancer cachexia revealed that ghrelin led to significant increase in weight and appetite (11, 60, 61). Another report by Nearry *et al.* showed that ghrelin infusion increased food intake of cachexic patients (62). In contrast, Strasser *et al.* reported opposite findings (63). As we have indicated, however, in many investigations on cancer cells treated with ghrelin, a proliferative effect was demonstrated and ghrelin possibly stimulated tumor growth. We also showed a correlation between ghrelin expression and depth of tumor invasion and advanced staging. A long-term clinical trial is required to determine if ghrelin treatment indeed stimulates tumor growth.

In conclusion we showed for the first time that some ESCCs express ghrelin and this was correlated with different clinicopathological findings. In the present study we suggest an important role of ghrelin for the tumor growth in ESCC. However, further investigation is required to establish the clinical significance of ghrelin as a biomarker or a therapeutic target in ESCC.

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ORIGINAL ARTICLE – THORACIC ONCOLOGY

The Usefulness of Neoadjuvant Chemoradiation Therapy for Locally Advanced Esophageal Cancer with Multiple Lymph-Node Metastases

Hiroshi Okumura, MD, PhD[1], Yasuto Uchikado, MD, PhD[1], Itaru Omoto, MD[1], Yoshiaki Kita, MD, PhD[1], Ken Sasaki, MD, PhD[1], Takaaki Arigami, MD, PhD[1], Yoshikazu Uenosono, MD, PhD[1], Daisuke Matsushita, MD[1], Yoshiyuki Hiraki, MD, PhD[2], Tetsuhiro Owaki, MD, PhD[3], Sumiya Ishigami, MD, PhD[1], and Shoji Natsugoe, MD, PhD[1]

[1]Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical Sciences, Kagoshima University, Kagoshima, Japan; [2]Department of Radiology, Graduate School of Medical Sciences, Kagoshima University, Kagoshima, Japan; [3]Education Center for Doctors in Remote Islands and Rural Areas, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan

ABSTRACT

Background. The prognosis of patients with esophageal squamous-cell cancer (ESCC) and multiple lymph-node metastases is quite poor. We examined whether neoadjuvant chemoradiation therapy (CRT) has a beneficial effect in such patients.

Methods. A total of 50 consecutive patients with T3–4 tumors and without organ metastases were prospectively enrolled. Of those patients, 20, who had four or more nodal metastases, underwent neoadjuvant CRT (CRT group), and the remaining 30 patients, who had three or fewer nodal metastases, underwent surgery alone (surgery group). CRT consisted of 5-fluorouracil plus cisplatin and 40 Gy of radiation. The groups' clinical outcomes were compared.

Results. Surgery was performed in 48 patients: all enrolled patients except for 2 who had organ metastasis after CRT. In the CRT group, the number of patients with pathological complete response was observed in 8 patients (44 %), mean nodal metastases number was changed from 8.2 to 2.6 and 9 patients had pN0. The 3-year survival rate was 76 % in the CRT group (4 patients relapsed) and 68 % in the surgery group (8 patients relapsed), which is not a statistically significant difference ($P = 0.61$).

Conclusions. Neoadjuvant CRT is beneficial for locally advanced ESCC with four or more lymph-node metastases.

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H. Okumura, MD, PhD
e-mail: hokumura@m.kufm.kagoshima-u.ac.jp

The prognosis is poor for patients with esophageal squamous-cell cancer (ESCC) with multiple lymph-node metastases. In particular, the prognosis in patients with four or more lymph-node metastases is significantly poorer than in those with three or fewer.[1] Preoperative endoscopic ultrasonography (EUS) is useful to diagnose clinical lymph-node metastases, with results that correlate well with pathological diagnoses.[1,2] Chemoradiation therapy (CRT) has been investigated since the 1980s for the treatment of ESCC and is one of the most useful treatments available; however, it remains controversial whether neoadjuvant CRT provides a significant advantage.[3–5]

We hypothesized that neoadjuvant CRT might be less useful for those patients who could be cured with surgery alone. In this study, we examined the effect of neoadjuvant CRT on patients with ESCC and four or more lymph-node metastases and compared the outcome with that of patients with three or fewer nodal metastases treated with surgery alone.

MATERIALS AND METHODS

Patients

The study included 130 consecutive ESCC patients who were treated at the Department of Surgical Oncology and Digestive Surgery of Kagoshima University Hospital

TABLE 1 Pretreatment clinicopathologic features

Characteristics	CRT group ($n = 20$)	Surgery group ($n = 30$)	P value
Gender (male/female)	20/0	30/0	NS
Age (years)	64.0 ± 7.1	66.4 ± 9.0	NS
cT3/T4	16/4	29/1	0.05
cN0/N1/N2/N3	0/0/12/8	7/8/15/0	<0.001
c-Number of metastatic LN	8.2 ± 5.3	1.9 ± 1.2	<0.001
cM0/M1	11/9	25/5	0.03
cStage II/III/IV	0/11/9	9/16/5	0.008

CRT chemoradiation therapy, *NS* not significant, *c* clinical

between January 2010 and December 2012. All patients had been preoperatively diagnosed and staged by esophagoscopy with biopsy, barium study, computed tomography (CT) scan of the neck, chest and abdomen, ultrasonography (US) of the neck and abdomen, EUS, and positron emission tomography (PET). Of these 130 patients, 50 had a T3 or T4 tumor without organ metastasis: 20 of these patients had four or more lymph-node metastases and underwent neoadjuvant CRT followed by curative surgery (CRT group); the other 30 patients, who had three or fewer lymph-node metastases, were treated with curative surgery only (surgery group). Patients in both groups underwent esophagectomy with lymph-node dissection, and the reconstruction was performed by cervical esophagogastric anastomosis using a gastric tube. In the CRT group, surgery was performed 4–6 weeks after completing CRT. After all patients had given their informed consent, the resected specimens were collected. The specimens were classified according to the International Union against Cancer tumor-node-metastasis (TNM) classification system.[6] Patients in the CRT group did not undergo adjuvant chemotherapy because their immune system was weakened by multimodal treatment; however, 17 patients who had pathological lymph nodes metastases in the surgery group underwent adjuvant chemotherapy consisting of docetaxel, cisplatin plus 5-fluorouracil. All patients were followed up with CT every 3 months after discharge, US every 6 months, and endoscopy every 6–12 months. Follow-up data after surgery were available for all patients, with a median follow-up period of 21 months (range 7–37 months). Mode of recurrence was categorized as lymph node, hematogenous, or local. For patients whose disease relapsed, CRT or chemotherapy was performed according to the relapse site and condition of patients. The pretreatment clinicopathologic features of the study group are summarized in Table 1. All M1 tumors were due to distant lymph-node metastases. The mean number of lymph-node metastases in the CRT group and the surgery group was 8.2 and 1.9, respectively. Thus, the CRT group had significantly more lymph-node metastases and more advanced stage. The CRT group also tended to have cT4 tumors. In the surgery group, 1 patient with cT4 had suspected tracheal invasion, but that was curatively removed and final diagnosis was pT3. There were 2 patients with pT4 invaded to lung and pleura that were curatively resected. The study was approved by the Institutional Review Board of Kagoshima University and performed according to the Helsinki Declaration.

Chemoradiation Therapy

A total radiation dose of 40 Gy was applied: 2 Gy fractions were delivered 5 days per week for 4 weeks to the mediastinum and neck. Supraclavicular to lower mediastinal lymph-node as well as cardiac to celiac lymph-node areas were irradiated as a long T-shaped field for upper to lower thoracic tumors, and perigastric LN areas were additionally irradiated for lower tumors. In the same period, chemotherapy was performed intravenously using 2 anticancer agents: cisplatin (7 mg/m^2 over 2 h) and 5-FU (350 mg/m^2 over 24 h).[1] Tumor response was evaluated by the Response Evaluation Criteria in Solid Tumors version 1.1.[7] The clinical response to CRT was evaluated by the findings of esophagography, esophagoscopy, US, EUS, CT, and PET for the lymph-node metastases. The clinical criteria for the response of target lesions were: complete response (CR), the disappearance of all target lesions as well as secondary changes associated with the tumors; partial response (PR), at least a 30 % decrease in the sum of the greatest dimensions of target lesions, relative to the baseline sum of greatest dimensions; progressive disease (PD), at least a 20 % increase in the sum of greatest dimensions of target lesions, relative to the smallest sum of greatest dimensions recorded since the treatment started; and stable disease (SD), neither PR nor PD. The histological criteria for the response of CRT were: grade 0, neither necrosis nor cellular or structural changes can be seen throughout the lesion; grade 1, necrosis or disappearance of the tumor is present in no more than two-thirds of the whole lesion; grade 2, necrosis or disappearance of the tumor is present in more than two-thirds of the whole lesion but viable tumor cells remain; and grade 3, the whole lesion falls into necrosis and/or is replaced by fibrosis, with or without granulomatous changes. No viable tumor cells are observed.[8,9] CRT was judged to be effective in patients whose histological response was grade 2 or 3, but ineffective in patients whose histological response was grade 0 or 1. Adverse events were diagnosed according to Common Terminology Criteria for Adverse Events v4.0.

Statistical Analysis

Statistical analysis of group differences was performed using the χ^2 test and t test. The Kaplan–Meier method was used for survival analysis, and differences in survival were estimated using the log-rank test. The P values in this study were 2-sided, and $P < 0.05$ was considered to indicate statistical significance. All statistical analyses were performed using the software package StatView version 5.0 (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Effects of Chemoradiotherapy

Of the 50 patients, surgical treatment was performed in 48, excluding 2 patients from the CRT group with bone or liver metastasis, although all 20 patients in the CRT group completed CRT. Grade 3 adverse effects comprised appetite loss ($n = 1$), leukopenia ($n = 2$), and anemia ($n = 1$). No serious complications requiring cessation of CRT were encountered. The number of patients with CR, PR, SD, and PD after CRT was 2, 11, 4, and 3, respectively. The number of patients with grades 1, 2, and 3 histological response was 8, 2, and 8, respectively.

Pathological Findings Table 2 summarizes the pathological diagnoses for the study groups. There were no significant differences in pN, p-number of lymph-node metastasis, and pM between the CRT group and the surgery group. Unlike the pretreatment diagnoses reported in Table 1, the tumors in the surgery group had deeper wall invasion and more advanced stage than those in the CRT group. The mean number of nodal metastases in the CRT group changed from 8.2 to 2.6 after CRT and 9 patients became pN0; the surgery group did not see a significant change (from 1.9 to 2.4) (Tables 1, 2).

Clinical Outcome

No hospital deaths were reported in the study. Anastomotic leakage was found in 3 patients (16 %) from the CRT group and in 4 (13 %) from the surgery group. Serious pneumonia with mechanical ventilation was reported in 1 patient (6 %) from the CRT group, and thoracic empyema was experienced by 1 patient (3 %) from the surgery group. The incidence of morbidity was not significantly different between the 2 groups.

Relapses occurred in 4 patients (22 %) in the CRT group and in 8 patients (26 %) in the surgery group. The mode of initial recurrence in the CRT group was lymph node ($n = 1$), hematogenous ($n = 2$), and local ($n = 1$). In the surgery group, mode of recurrence was lymph node ($n = 4$), hematogenous ($n = 3$), and local ($n = 1$) (Table 3). During the study period, 3 patients died of relapsed disease and 1 in the CRT group died of pneumonia. In the surgery group, all 8 patients with recurrence died of esophageal cancer. The 3-year survival rate was 76.4 % in the CRT group and 68.2 % in the surgery group ($P = 0.61$) (Fig. 1). Furthermore, the survival rate of patients underwent surgery in Ref.[1] that we previously reported was estimated and compared with that of CRT group. The 3-year survival rates of patients with three and fewer and four or more lymph nodes metastases diagnosed by ultrasound and endoscopic ultrasound before surgery

TABLE 2 Pathological diagnoses

Characteristics	CRT group ($n = 18$)	Surgery group ($n = 30$)	P value
pT0/T1/T2/T3/T4	8/4/0/6/0	0/7/4/17/2	0.001
pN0/N1/N2/N3	9/4/4/1	13/9/3/5	NS
p-Number of metastatic LN	2.6 ± 4.4	2.4 ± 3.6	NS
pM0/M1	15/3	26/4	NS
pStage 0/I/II/III/IV	5/2/1/7/3	0/4/12/11/3	0.008

CRT chemoradiation therapy, *NS* not significant, *p* pathological

TABLE 3 Mode of initial recurrence

	CRT group ($n = 18$)	Surgery group ($n = 30$)
Number of recurrence	4	8
Mode of recurrence		
Lymph node	1	4
Hematogenous	2	3
Local	1	1

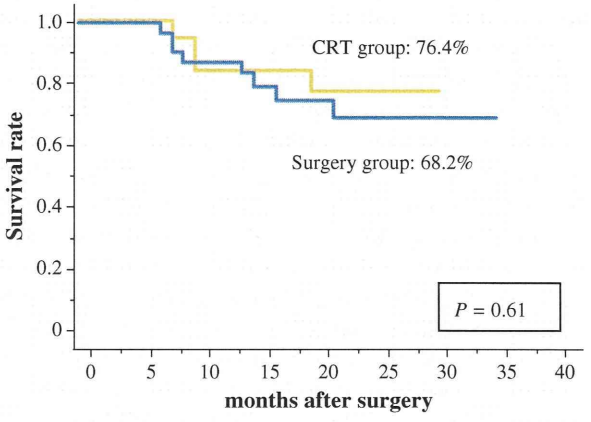

FIG. 1 Survival analyses. The 3-year survival ratios were 76.4 and 68.2 % for the CRT group and surgery group, respectively. There was no significant difference between the 2 groups

were 54.9 and 16.0 %, respectively, that were significantly worse than that of the CRT group in this study.

DISCUSSION

Lymph-node metastasis is the most important prognostic factor for esophageal cancer, so its accurate diagnosis prior to surgical treatment is indispensable.[1,10] There is a significant difference in 5-year survival between patients with three or fewer and those with four or more metastases.[1] Therefore, we usually determine the number of lymph-node metastases using US and EUS, whose results correlate well with pathological diagnoses.[1,2,11] For ESCC patients treated with CRT, it is important to correctly diagnose clinical nodal status in order to ensure that an appropriate radiation field is set for CRT and subsequently to perform surgical treatment.[10,11]

Although many surgeons, radiologists, and oncologists have made efforts to improve the survival of ESCC patients, survival benefit of neoadjuvant CRT remains controversial. Some retrospective studies have shown prognosis to be improved due to the effects of neoadjuvant CRT, while some randomized controlled studies have found no significant survival difference between treatment with neoadjuvant CRT and surgery alone.[3,12–17] In our previous study, pN0 patients had better survival even when they had clinical lymph-node metastases.[10] Taken together, these results suggest that neoadjuvant CRT might be less useful for patients with a few lymph-node metastases, who could be cured by surgery alone, and more useful for the patients with four or more lymph-node metastases who cannot be cured with surgery alone and need multimodal treatment. In this prospective trial, neoadjuvant CRT succeeded in decreasing the number of lymph-node metastases: there was no statistically significant difference in survival between the CRT group and the surgery group, which indicates an improvement in survival in the CRT group. Accurate preoperative lymph-node diagnosis enables ESCC patients to receive targeted treatment to reduce multiple lymph-node metastases and thus allows curative surgery.

Of the 4 patients who had hematogenous recurrent disease in the CRT group, 1 was judged as CR. Previous experience has shown that pN0 patients tend to have hematogenous rather than lymph-node recurrence.[10] Meguid et al.[18] also reported that most recurrences of esophageal cancer after neoadjuvant therapy and surgery are distant, even in patients with complete pathologic response. This is one of the most important problems facing clinicians performing CRT on esophageal cancer patients, and we should clarify the mechanisms by which good responders have hematogenous relapses and investigate how to modify the treatment strategy to avoid this problem.

Nowadays, preoperative neoadjuvant chemotherapy is recommended for patients with cStage II or III ESCC patients in Japan; the 5-year overall survival was 55 % in patients who received preoperative chemotherapy with cisplatin plus 5-fluorouracil, which was superior to 43 % in patients who received postoperative chemotherapy.[19] However, survival rate was still unsatisfactory; therefore, we are exploring a new treatment strategy by trying to modify the use of CRT, which has a powerful tumor-shrinking effect, in order to improve the survival of ESCC patients.

In conclusion, neoadjuvant CRT was beneficial for patients with locally advanced esophageal cancer with four or more lymph-node metastases.

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Clinical and biological impact of cyclin-dependent kinase subunit 2 in esophageal squamous cell carcinoma

YOSHIAKI KITA[1*], YUKA NISHIZONO[1*], HIROSHI OKUMURA[1], YASUTO UCHIKADO[1], KEN SASAKI[1], MASATAKA MATSUMOTO[1], TETSURO SETOYAMA[1], KIYONORI TANOUE[1], ITARU OMOTO[1], SHINICHIRO MORI[1], TETSUHIRO OWAKI[1], SUMIYA ISHIGAMI[1], HIROSHI NAKAGAWA[2], FUMIAKI TANAKA[3], KOSHI MIMORI[3], MASAKI MORI[4] and SHOJI NATSUGOE[1]

[1]Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medicine, Kagoshima University, Kagoshima 890-8520, Japan; [2]Division of Gastroenterology, Department of Medicine, Abramson Cancer Center, Perelman School of Medicine, Philadelphia, PA 19104, USA; [3]Department of Surgery, Bepppu Hospital, Kyushu University, Beppu 874-0838; [4]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Suita 565-0871, Japan

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Abstract. Cyclin-dependent kinase subunit 2 (CKS2) is a cyclin-dependent kinase subunit (CKS) family member that participates in cell cycle regulation. Few studies have investigated its involvement in esophageal squamous cell carcinoma (ESCC). The aim of the present study was to assess the clinical significance of CKS2 in ESCC. We used immunohistochemistry to study the clinicopathologic significance of CKS2 protein expression in 121 patients with ESCC. Using real-time reverse transcriptase-polymerase chain reaction (RT-PCR), we examined the expression of *CKS2* mRNA in tumors and the corresponding normal esophageal tissues that were obtained from 62 patients. Finally, siRNA-mediated attenuation of CKS2 expression was examined *in vitro*. CKS2 protein expression was significantly correlated with depth of tumor invasion, clinical stage, lymphatic invasion and distant metastasis (p=0.033, 0.028, 0.041 and 0.009, respectively). *CKS2* mRNA expression was higher in cancer tissue than in corresponding normal tissue (p<0.001). Patients with positive-CKS2 protein expression had a poorer five year survival frequency than patients who did not express CKS2 protein (p=0.025). *In vitro*, siRNA-mediated suppression of CKS2 slowed the growth rate of ESCC cells compared to control cells (p<0.001). The evaluation of CKS2 expression is useful for predicting the cause of malignant tumors and the prognosis of patients with ESSC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive cancers of the gastrointestinal tract. It frequently progresses to invasion and metastasizes to the lymph nodes and other organs, and once metastatic, prognosis is poor (1,2). Although the biological factors affecting the malignant potential of ESCC have been identified, the molecular mechanisms underlying its progression have not been completely elucidated. Finding a cure for this intractable malignancy depends on the identification of genetic and molecular markers of malignancy potential, that may serve as specific treatment targets. However, the regulation of complex processes over multiple events precludes the identification of practical markers for carcinogenesis, tumor progression and metastasis.

Cyclin-dependent kinase subunit (CKS) proteins are small (9-kDa) cyclin-dependent kinase (Cdk)-interacting proteins that are expressed in all eukaryotic lineages. Those proteins include the highly conserved paralogs CKS1 and CKS2 in mammals (3). Both CKS1 and CKS2 consist of 79 amino acids and they possess 81% homology. The structural basis for the CKS-Cdk interaction is well understood, as the three dimensional configuration of the heterodimeric complex has been determined by X-ray diffraction crystallography (4). In addition, genetic analysis of CKS protein function in mammals is quite advanced. CKS1 is a specific co-factor that is necessary for the degradation and ubiquitination of p27 by SCF^{Skp2}. Human CKS1 binds to Skp2 and increases the binding of threonine 187-phosphorylated p27 to Skp2 (5,6). On the other hand, Cks2 is essential for meiosis. This phenotype results

Correspondence to: Dr Yoshiaki Kita, Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890-8520, Japan
E-mail: north-y@m.kufm.kagoshima-u.ac.jp

*Contributed equally

Abbreviations: ESCC, esophageal squamous cell carcinoma; CKS, cyclin-dependent kinase subunit; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-polymerase chain reaction

Key words: cyclin-dependent kinase subunit 2, esophageal squamous cell carcinoma, prognosis, biomarker