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Effective Mediastinal Lymphadenectomy for Esophageal Cancer Using Slender Tracheal Forceps in Prone Position Thoracoscopic Esophagectomy

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Abstract. *Background/Aim: Adequate mediastinal lymphadenectomy during thoracoscopic esophagectomy (TE) requires an extensive operating field. In order to rectify this problem, we developed slender tracheal forceps that can pass through a 12-mm trocar. Patients and Methods: TE in the prone position was performed in 58 patients with esophageal cancer using slender tracheal forceps. Perioperative and postoperative clinical data were compared against those of 61 patients who underwent transthoracic open esophagectomy (OE). Results: The mean duration of thoracic manipulation was significantly longer in the TE than OE group: 226.2 versus 171.3 minutes (p<0.0001). Mean blood loss was significantly lower in the TE than OE group: 39.9 versus 176.8 g (p<0.0001). A mean of 23.4 mediastinal lymph nodes were dissected in the TE group and 25.1 in the OE group. The timing of extubation tended to be earlier in the TE group. Postoperative morbidity did not differ between groups. Conclusion: Slender tracheal forceps are acceptable for fine mediastinal lymphadenectomy in thoracoscopic surgery for esophageal cancer, and the technique could contribute to development of minimally invasive surgery.*

Surgery for esophageal cancer is time-consuming and complicated. The three most common techniques for thoracic esophagectomy are the transhiatal approach, Ivor Lewis esophagectomy (right thoracotomy and laparotomy), and the McKeown technique (right thoracotomy followed by laparotomy and neck incision with cervical anastomosis) (1). In Japan, extended transthoracic esophagectomy with three-field lymphadenectomy (cervical, mediastinal and abdominal) with the McKeown technique has been performed for the past three decades (2). Although the prognosis of patients with esophageal cancer has improved as a result of such extensive surgery, postoperative morbidity and mortality are important problems that remain to be resolved (3, 4).

Recently, thoracoscopic esophagectomy (TE) has become more popular among surgeons, with an expectation that it will become a minimally invasive procedure (5). Although its consideration as a minimally invasive surgical technique is controversial (6), it seems to be equal or superior to conventional transthoracic surgery for anatomical recognition because of its magnified field of view (7). For TE to become a standard operating procedure, sufficient lymphadenectomy is required.

In esophageal cancer surgery, removal of the upper mediastinal lymph nodes, especially those of the right and left recurrent nerves, is important because of their high rate of metastasis (8). For adequate mediastinal lymph node dissection, an extensive operating field is required, and appropriate devices are needed to achieve this.

At our Institute, TE in the prone position has been performed for early-stage cancer. This approach is beneficial for surgeons because an adequate surgical field can be obtained without assistance, and it can provide surgeons with superior ergonomics for the left lateral position (9). However, the difficulty of lymph node dissection in the upper mediastinum, especially on the left side of the trachea, has been reported (10). In order to rectify this problem, we developed slender tracheal forceps that can pass through a 12-mm trocar and create an adequate operating field.

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Key Words: Esophageal cancer, thoracoscopic esophagectomy, lymphadenectomy, minimal invasive surgery, prone position.

In this study, we investigated the effectiveness of this forceps and its suitability in TE.

Patients and Methods

Patients. From January 2011 to October 2014, 172 patients with esophageal cancer underwent esophagectomy at Dokkyo Medical University Hospital, Japan. We excluded patients who underwent transhiatal esophagectomy (40 cases), salvage esophagectomy (eight cases) and cervical esophagectomy including laryngopharyngoesophagectomy (five cases). Patients who underwent TE or transthoracic subtotal esophagectomy were eligible. TE with mediastinal lymphadenectomy in the prone position was performed in 58 patients with thoracic or abdominal esophageal cancer (TE group). The selection criteria for TE were as follows: no previous thoracic surgery; no possibility of severe pleural adhesion; and no previous (chemo-) radiotherapy of the esophagus. For the first 40 cases, this procedure was performed in patients with clinical T1/T2, N0 esophageal cancer. After that, the indications were extended to T3 and N1-3. In the same period, 61 patients underwent transthoracic open esophagectomy (OE group). The indications for transthoracic open esophagectomy were clinical T1-4a/N0-3 esophageal cancer (Table I).

The patients were staged according to the TNM classification (seventh edition) of the American Joint Committee on Cancer and the International Union Against Cancer (11). Informed consent for the operations was obtained from all patients according to our institutional guidelines.

Thoracoscopic surgery. The patients were intubated with a single-lumen endotracheal tube, and a blocking balloon was inserted into the right main bronchus to deflate the right lung. The patients were placed in the prone position. The first thoracoscopic port (12-mm Xcel port; ETHICON, New Brunswick, USA) was inserted at a posterior axillary line at the seventh intercostal space, and inflated with CO2 at 6 mm Hg, which collapsed the right lung. Next, another 12-mm port was inserted at the scapular angle line at the ninth intercostal space. This port was used as the main observation port. Thereafter, three ports were arranged as follows: a 12-mm port at the middle axillary line at the third intercostal space; a 12-mm port at the posterior axillary line at the fifth intercostal space; and a 5-mm port at the scapular angle line at the seventh intercostal space. TE was started with mobilizing the upper esophagus with the regional lymph nodes. In order to mobilize the upper esophagus, the azygos vein and the right bronchial artery were divided. The upper mediastinal pleura was dissected and the upper thoracic esophagus, right main branch of the vagal nerve and right subclavian artery were exposed. After that, the right recurrent nerve lymph nodes were dissected up to the thyroid gland. We utilized a slender tracheal forceps (total length: 400 mm, length excluding handgrip: 290 mm, length of head: 43 mm, maximum diameter of head: 8 mm) that we developed ourselves (Figure 1). Moderate traction of the subclavian artery using this forceps allowed effective visualization of the surgical site (Figure 2). The upper thoracic esophagus was circumferentially mobilized and taped to allow moderate retraction. This tape was grasped by a Mini Loop Retractor II (COVIDIEN JAPAN, Tokyo, Japan) inserted through the chest wall at the inside of the right scapula. The upper thoracic esophagus was retracted to the dorsal side by pulling up the retractor. The adjacent trachea was rolled to the right and ventrally by the tracheal forceps (Figure 3). Tissue including the left recurrent nerve lymph nodes was radically dissected from this extended surgical site. In the middle mediastinum, the bilateral esophageal branch of the vagal nerve was divided, while the pulmonary branches were preserved. The subcarinal and bilateral main bronchial lymph nodes were dissected with the middle esophagus. In the lower mediastinum, the esophagus and regional lymph nodes were dissected along the layer that exposed the pericardium, descending aorta and left mediastinal pleura. After complete dissection of the thoracic esophagus and regional lymph nodes, a 24 Fr chest drainage tube was inserted through the 12-mm port site at the ninth intercostal space.

Table I. *Patient characteristics of thoracoscopic esophagectomy (TE) and transthoracic open esophagectomy (OE) groups.*

Factor	TE group (n=58)	OE group (n=61)	*p*-Value
Age (mean±SD)	65.95±8.23	63.82±9.36	0.1911
Gender (M/F)	49/9	49/12	0.7220
Tumor location			0.9947
Upper thoracic	7	7	
Mid thoracic	32	34	
Lower thoracic	19	20	
Depth of tumor invasion			<0.0001
T0	0	1	
T1	43	6	
T2	7	6	
T3	7	40	
T4	1	8	
Lymph node metastasis			<0.0001
N0	44	20	
N1	8	17	
N2	4	13	
N3	2	11	
Distant metastasis			0.9713
M0	57	60	
M1 (Lym)	1	1	
Stage			<0.0001
0	0	1	
I	42	8	
II	8	10	
III	7	41	
IV	1	1	
Histology			0.0381
Squamous cell carcinoma	49	53	
Barrett adenocarcinoma	5	1	
Adenocarcinoma	0	5	
Other	4	2	
Chemotherapy (yes/no)	4/54	26/35	<0.0001

F: Female; M: male; SD: standard deviation; Lym: lymph node.

Perioperative and postoperative clinical course. The duration and blood loss of thoracic surgery were measured in the TE and OE groups, along with the number of dissected mediastinal lymph nodes. Additionally, we noted the duration of intubation, intensive care unit (ICU) stay, and postoperative hospital stay. Postoperative morbidity was assessed according to the Clavien–Dindo

Figure 1. *Slender tracheal forceps made at our institution. A: Complete view of the forceps. B: Magnified view of the head of the forceps. The obtuse angle allows gentle exclusion and traction of the trachea. This is appropriate as an artery or nerve exclusion forceps.*

classification (12), and complications greater than grade II were regarded as significant. Surgical mortality (Clavien–Dindo grade V) included in-hospital deaths (by postoperative day 90).

Statistical analysis. Categorical analysis of variables was performed using either the Chi-square of Fisher's exact test, as appropriate. Continuous data were compared with the Mann–Whitney *U*-test. Differences were considered to be significant if the *p*-value was less than 0.05. All statistical analyses were carried out using R software (version 3.1.1).

Results

Perioperative course. The surgical outcomes of esophagectomy are described in Table II. The duration of thoracic manipulation in the TE group was significantly longer than that in the OE group ($p<0.0001$) at a mean of 226.2 minutes compared to 171.3 minutes in the OE group. Mean blood loss was 39.9 g in the TE group, compared with 176.8 g in the OE group ($p<0.0001$). Although there was a tendency towards less frequent blood transfusion in the TE group, it was not significant. There were a mean 23.2 mediastinal lymph nodes dissected in the TE group and 24.8 in the OE group. There was no significant difference between the groups ($p=0.1083$). There was a mean of 4.2 left recurrent nerve lymph nodes dissected in the TE group and 4.5 in the OE group, but this difference was not significant ($p=0.4885$). In the TE group, the mean duration of intubation was 0.03 days, compared with 0.33 days in the OE group. Although the difference was not significant, there was a tendency toward earlier extubation in the TE group ($p=0.0667$). With regard to ICU stay and postoperative hospital stay, there were no significant differences between the groups ($p=0.4980$ and $p=0.2066$, respectively).

Postoperative morbidity and mortality. There was no postoperative mortality. The incidence of postoperative morbidity did not differ significantly between the groups. Each complication was analyzed according to the Clavien–Dindo classification (Table III). With regard to recurrent nerve palsy, grade I or more dysfunction is shown. For other complications, including pneumonia, arrhythmia, anastomotic leakage and wound infection, those over grade II are shown. The most frequent complications for the whole cohort were recurrent nerve palsy and anastomotic leakage. The two groups did not differ significantly in regard to frequency of complications. Wound infection did not occur in these two groups. One patient each in the OE group had pyothorax and pleuritis.

Figure 2. *Photograph of lymph node dissection of the right recurrent nerve (white arrow) using a slender tracheal forceps. By excluding the right subclavian artery (black arrow) ventrally, a sufficient surgical view is obtained.*

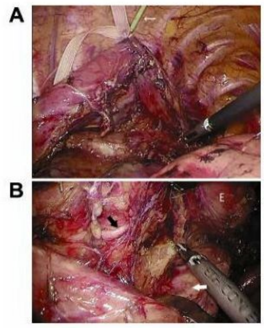

Figure 3. *Photograph of lymph node dissection of the left recurrent nerve (black arrow) using a slender tracheal forceps and Mini loop retractor II. A: Mediastinal tissue, including left recurrent nerve, was extended by pulling the upper esophagus to the dorsal side using a Mini Loop Retractor II. (B) As a result of traction of the trachea to the ventral and right side, clear view around the left recurrent nerve is obtained. White arrow, trachea; E, esophagus.*

Table II. *Surgical outcomes of thoracoscopic esophagectomy (TE) and transthoracic open esophagectomy (OE) groups.*

	TE group (n=58)	OE group (n=61)	*p*-Value
Duration of thoracic manipulation (mean±SD) (min)	226.22±35.81	171.25±35.40	<0.0001
Blood loss in thoracic cavity (mean±SD) (g)	39.86±56.10	176.82±131.07	<0.0001
Blood transfusion (yes/no)	4/54	11/50	0.0623
No. of dissected mediastinal nodes (mean±SD)	23.17±5.64	24.82±5.47	0.1083
No. of dissected left recurrent nerve nodes (mean±SD)	4.24±1.72	4.49±2.17	0.4885
Intubation time (d)	0.034±0.184	0.328±1.193	0.0667
ICU stay (d)	1.000±0.000	1.082±0.918	0.4980
Hospital stay (d)	16.8	20.8	0.2066

ICU: Intensive care unit; SD: standard deviation.

Discussion

Thoracoscopic surgery for esophageal cancer is often performed as a type of minimally invasive surgery (13-17). Although its importance as a minimally invasive procedure has been controversial to date, the reduction in blood loss that results from being able to recognize fine structure is a merit of thoracoscopic surgery (18). Additionally, precise lymph node dissection may be possible because of the ability to view the fine structure (7). Recently, many institutions have introduced TE, especially in Japan and East Asia. However, more effective devices to enable sufficient lymphadenectomy and less invasiveness will be required if thoracoscopic surgery is to become a gold standard method of esophagectomy.

Among the mediastinal lymph nodes, those of the right and left recurrent nerves are important targets for lymphadenectomy because metastasis is frequent in these areas (19-21). In order to create an adequate operating field, we developed slender tracheal forceps.

TE is performed with the patient in the left lateral decubitus position (22-24) or prone position (9, 25-27). At our institute, we select to perform TE with the patient in the prone position because of its convenience for effective visualization of the mediastinum, and because accumulation of blood in the operating field is rare. However, the prone position is thought to be inferior to the left lateral position regarding left upper mediastinal lymph node dissection because effective visualization of the surgical field is difficult. Our method using slender tracheal forceps and Mini Loop Retractor II have an advantage of visualization of the surgical field derived from retraction and extension of the trachea and esophagus. For retraction of the trachea, endoscopic gauze or dissectors are generally used. However, it is easy for these instruments to slip on the membranous portion of the trachea. Therefore, frequent modification of retraction is necessary. There is rarely a need for modification of tracheal exclusion using our method. With regard to the Mini Loop Retractor II, which is a type of needle device (28), it is useful to pull the esophagus to the dorsal side, and to make a good operating field. Using these devices fully, an effective operating field is exposed for sufficient upper mediastinal lymphadenectomy.

Table III. *Postoperative morbidity of thoracoscopic esophagectomy (TE) and transthoracic open esophagectomy (OE) groups.*

	TE group (n=58)	OE group (n=61)	*p*-Value
Morbidity[a]	21 (36.2%)	26 (42.6%)	0.4742
Recurrent nerve palsy[b]	12 (20.7%)	13 (19.7%)	0.9337
Pneumonia	3 (5.2%)	4 (6.6%)	0.7483
Arrhythmia	3 (5.2%)	3 (4.9%)	0.9495
Anastomotic leakage	8 (13.8%)	9 (14.8%)	0.8810
Wound infection	0 (0%)	0 (0%)	-
Other	0 (0%)	2 (3.3%)	-

[a]Over Clavien–Dindo classification grade II; [b]greater than Clavien–Dindo classification grade I.

Apart from effective lymph node dissection, prevention of postoperative mortality is a requirement of thoracoscopic surgery. Thoracoscopic surgery can minimize damage to the thoracic wall and preserve the respiratory muscles (7). Additionally, the magnified field of view allows for preservation of the minute vessels around the trachea or bronchus. In particular, a lateral longitudinal anastomosis, derived from the inferior thyroid artery, subclavian artery, internal thoracic artery, tracheoesophageal artery, and costocervical trunk, is important for tissue blood supply in the trachea (29). For the thoracoscopic technique, we can confirm lateral longitudinal anastomosis and dissect the lymph nodes around the trachea without injury. Our slender tracheal forceps are helpful for preservation of the lateral longitudinal anastomosis.

The point needing improvement for TE is shortening the long operative time. One report has highlighted the necessity for a specialist team comprising three experts (namely a surgeon, an assistant, and an endoscopist) for smooth TE (27). In our study, the operating time for mediastinal manipulation was significantly longer than that required for transthoracic surgery. One reason for this is that endoscopists are changed at regular intervals (*e.g.* every 3 months). Unlike transthoracic surgery, the skill of the endoscopist is important in thoracoscopic surgery. Optimal visualization depends on the endoscopist securing an effective visual field. Therefore, the education of endoscopists is one of the most important issues for effective thoracoscopic surgery. If standardization of the thoracoscopic procedure is achieved by skillful endoscopists and effective devices for surgical field extension are available, shorter operating times will be possible.

Conclusion

In conclusion, our procedure using slender tracheal forceps is acceptable for fine mediastinal lymphadenectomy in thoracoscopic surgery for esophageal cancer. We believe that the dissemination of these procedures could contribute to further development of minimally invasive surgery for esophageal cancer.

Conflicts of Interest

Masanobu Nakajima and other co-authors have no conflict of interest.

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Efficacy of system L amino acid transporter 1 inhibition as a therapeutic target in esophageal squamous cell carcinoma

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System L amino acid transporter 1 (LAT1) is highly expressed in various types of human cancer, and contributes to cancer growth and survival. Recently, we have shown that LAT1 expression is closely related to the growth and aggressiveness of esophageal cancer, and is an independent marker of poor prognosis. However, it remains unclear whether LAT1 inhibition could suppress esophageal cancer growth. In this study, we investigated the tumor-suppressive effects of the inhibition of LAT1. Both LAT1 and CD98, which covalently associates to LAT1 on the membrane, were expressed in human esophageal cancer cell lines KYSE30 and KYSE150. Quantitative PCR analysis showed that the expression of LAT1 was much higher than other subtypes of LAT. A selective inhibitor of LAT, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), suppressed cellular uptake of L-^{14}C-leucine and cell proliferation in a dose-dependent manner. It also suppressed phosphorylation of mammalian target of rapamycin, 4E-BP1, and p70S6K protein, and induced cell cycle arrest at G_1 phase. These results suggest that suppression of both mammalian target of rapamycin signaling and cell cycle progression is involved in BCH-induced growth inhibition. In tumor-bearing mice, daily treatment with BCH significantly delayed tumor growth and decreased glucose metabolism, indicating that LAT1 inhibition potentially suppresses esophageal cancer growth *in vivo*. Thus, our results suggest that LAT1 inhibition could be a promising molecular target for the esophageal cancer therapy.

Esophageal cancer is one of the most common cancers and is highly lethal. In 2008 data reported by Jemal *et al.*,[1] approximately 480 000 new cases and 400 000 deaths occurred worldwide. Esophageal cancer is classified in cervical, thoracic, and gastroesophageal cancer; the major type in Asia is squamous cell carcinoma (SCC). Although the curative rate is improved by novel therapeutic approaches, 5-year survival of patients is still low.[2] Early detection of esophageal cancer is difficult because patients rarely experience subjective symptoms in the early stages of the cancer. In addition, as esophageal cancer easily metastasizes, patients often have advanced or metastatic disease on admission. Patients with unresectable disease are usually treated with chemotherapy such as a combination of cisplatin (CDDP) and 5-fluorouracil (5-FU). However, common chemotherapeutic agents are not curative, and then the prognosis after treatment remains dismal. Therefore, it is important to establish novel molecular targets for improvement of therapeutic efficacy in esophageal cancer.

Amino acid transporters are essential for not only normal cells but also growth and survival of cancer. System L amino acid transporter 1 (LAT1) is highly expressed in various primary human cancers and tumor cell lines.[3–13] It couples with CD98 on the plasma membrane for its functional expression, and sodium-independently transports neutral amino acid that has large side-chain.[14] LAT1 has significant roles for cancer growth and survival through efficient supply of amino acids and activation of mammalian target of rapamycin (mTOR) signaling.[3,15] The high expression of LAT1 is correlated with various biomarkers of malignancy, such as p53, Ki-67, and CD34, and could be an independent prognostic marker in prostate, breast, pancreatic, lung, tongue, and biliary tract cancer.[7–13] Therefore, the possibility of molecular-targeted therapy focused on LAT1 inhibition has been investigated in many cancers. A competitive inhibitor of LAT, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), or other inhibitors of LAT1 significantly inhibit cellular uptake of amino acids and mTOR phosphorylation, leading to cell cycle arrest and apoptosis *in vitro*, and delays the growth of tumor *in vivo*.[6,12,16–19]

Recently we have found that LAT1 is closely related to the expression of CD98, growth, angiogenesis, and glycolysis in surgically resected human esophageal cancer, and expression of LAT1 could be an independent prognostic marker of esophageal cancer.[20] However, the therapeutic efficacy of LAT1 inhibition against esophageal cancer remains unclear. In this study, we undertook both *in vitro* and *in vivo* studies to investigate the suppressive effects of LAT1 inhibition on the growth of esophageal cancer.

Materials and Methods

Cell culture. Human esophageal cancer cell lines, KYSE30 (JCRB0188) and KYSE150 (JCRB1095) were purchased from the Health Science Research Resources Bank (Osaka, Japan),[21] and routinely maintained in DMEM (Wako Pure Chemical Industries, Osaka, Japan) containing 10% heat-inactivated FBS (AusGeneX, Loganholme, Australia), penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) at 37°C in 5% CO_2, 95% air. HEK293-mock and HEK293-hLAT1 established by Khunweeraphong *et al.*[22] were routinely maintained in Eagle's minimum essential medium (Wako Pure Chemical Industries) containing 10% heat-inactivated FBS (AusGeneX), non-essential amino acids (Wako Pure Chemical Industries), penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) at 37°C in 5% CO_2, 95% air.

Quantification of mRNA. Quantitative PCR (qPCR) analysis was carried out to quantify the expression of LAT1, LAT2, LAT3, and LAT4 mRNA in KYSE30 and KYSE150. Total RNA was isolated from cells using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). The first-strand cDNA was synthesized from 0.5 μg total RNA with PrimeScript Reverse Transcriptase (Takara Bio, Shiga, Japan). Full-length DNA of LAT1, LAT2, LAT3, and LAT4 was synthesized from cDNA of KYSE150 with KOD-Plus-Neo (Toyobo, Osaka, Japan) under the following conditions: 98°C for 60 s; five cycles of 98°C for 10 s, 74°C for 60 s; five cycles of 98°C for 10 s, 72°C for 60 s; five cycles of 98°C for 10 s, 70°C for 60 s; 20 cycles of 98°C for 10 s, 68°C for 60 s; and a final extension at 72°C for 10 min. After purification with High Pure PCR Product Purification Kit (Roche Applied Science, Indianapolis, IN, USA), PCR products were analyzed using agarose gel electrophoresis and single band of each full-length DNA of LATs was detected. Concentration of the full-length DNA of LATs was determined with BioPhotometer (Eppendorf, Hamburg, Germany). The qPCR carried out using serial dilutions of known concentrations of full-length DNA of LATs as templates and generated standard curves. The quantity of LAT1, LAT2, LAT3, and LAT4 mRNA in KYSE30 or KYSE150 cells was determined from the standard curves. The qPCR condition was as follow: after incubating each cDNA sample with the primers (0.5 μM each) and Thunderbird SYBR qPCR Mix (Toyobo), amplification was carried out for 40 cycles (95°C for 15 s, 60°C for 30 s) with a Piko-Real thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). The sequences of specific primers for analysis are shown in Table S1.

Suppression of amino acid uptake into cells with LAT1 inhibition. Inhibition of amino acid transport by BCH (NARD Institute, Hyogo, Japan) was examined according to our previous report.[12] Briefly, cells (1.0×10^5 cells/well) were plated in 24-well plates and incubated in growth medium for 24 h. After the incubation, the cells were washed three times with Na^+-free HBSS. The cells were incubated in Na^+-free HBSS for 10 min at 37°C, then the supernatant was replaced by Na^+-free HBSS containing 1 μM L-^{14}C-leucine (PerkinElmer Life Sciences, Boston, MA, USA), and various concentrations of BCH (1, 3, 10, 30, 100, 300, 1000, or 3000 μM). At 1 min after treatment with L-^{14}C-leucine, uptake was terminated by removing the uptake solution followed by washing three times with ice-cold Na^+-free HBSS. Cells were solubilized with 0.1 N NaOH, and radioactivity was measured by liquid scintillation spectrometry (AccuFLEX LSC-7200; Hitachi Aloka Medical, Tokyo, Japan). The L-^{14}C-leucine uptake was shown as %dose, which was calculated from the following formula: %dose = radioactivity of the solubilized cells/radioactivity of added L-^{14}C-leucine × 100.

Suppression of cell proliferation with LAT1 inhibition. Cells were plated at a concentration of 1×10^3 cells/well in 96-well plates and incubated in the growth medium for 24 h. At first, in order to determine the effect of LAT1 inhibition on esophageal cancer, cells were treated with BCH (1, 3, 5, 10, 20, 30, 40, 50, or 100 mM) and incubated for 3 days. Next, the effect of LAT1 inhibition on the antitumor activity of CDDP (Eli Lilly, Indianapolis, IN, USA) or 5-FU (Kyowa Hakko Kirin, Shizuoka, Japan) was evaluated. Cells were incubated for 3 days with CDDP (0.3, 1, 0.5, 1, or 10 μM) or 5-FU (1, 10, 100, or 1000 μM) in the presence or absence of 30 mM BCH. Cells were then incubated with 0.5 mg/mL MTT for 4 h at 37°C. The resulting formazan was solubilized, and the absorbance was read at 590 nm with a microtiter plate reader (V_{max}; Molecular Devices, Sunnyvale, CA, USA).

Detection of lactate dehydrogenase release. Cells (1.0×10^4 cells/well) were incubated for 24 h in a 96-well culture plate and treated with BCH (1, 3, 5, 10, 20, 30, 40, 50, or 100 mM) and incubated for 24 h. At the end of incubation, supernatants were collected and the lactate dehydrogenase (LDH) content was measured using a Cytotoxicity Detection Kit (Roche Applied Sciences, Laval, Canada). Lactate dehydrogenase release is expressed as a percentage of total content, which was determined by lysing an equal amount of cells with 1% Triton X-100.

Cell cycle analysis. Cells were plated at a concentration of 2×10^5 cells in 100 mm dishes and incubated in the growth medium for 24 h. After incubation, cells were treated with growth medium or BCH (30 mM) for 24 and 48 h. After incubation, cells were harvested from the dish, washed with PBS, and fixed with 70% ethanol at −20°C for 24 h. Cells were then washed three times with PBS and stained with 1 mg/mL propidium iodide solution containing 1 mg/mL RNase. Cell cycle was analyzed using a flow cytometer (EC800; Sony, Tokyo, Japan).

Immunoblotting. Cells were dissolved in sample buffer (25% glycerin, 1% SDS, 62.5 mM Tris-Cl, 10 mM DTT) and incubated at 65°C (LAT1) or 95°C (CD98, β-actin, mTOR, p-mTOR, 4E-BP-1, p-4E-BP-1, p70S6K, and p-p70S6K) for 15 min. Aliquots of samples containing 40 μg protein were analyzed by 10% SDS-PAGE and transferred onto a PVDF membrane. Blots were incubated at 4°C overnight in 10 mM Tris–HCl, 100 mM NaCl, 0.1% Tween-20, pH 7.5 (TBST), with 5% skim milk (LAT1, CD98, and β-actin) or 1% BSA (mTOR, p-mTOR, 4E-BP-1, p-4E-BP-1, p70S6K, and p-p70S6K). Blots were then incubated with antibodies at 4°C overnight. Details of antibodies are described in Table S2.[23] After washing with TBST, the blots were incubated with HRP-conjugated anti-rabbit IgG antibody for 1.5 h at room temperature. The blots were further washed with TBST, and specific proteins were visualized by using ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ).

Antitumor effect of LAT1 inhibition in tumor-xenograft model. The animals were cared for and treated in accordance with the guidelines of the animal care and experimentation committee at our facility. Antitumor effect of LAT1 inhibition was examined as previously described.[12] Briefly, KYSE150 cells (1×10^7 cells) were s.c. inoculated into the flank of 5-week-old male BALB/c nude mice (CLEA Japan, Tokyo, Japan). After tumor volumes had reached approximately 50 mm^3, the mice were divided into a control group and a treatment group ($n = 10$). Saline or BCH (200 mg/kg) was given i.v. once daily from the day of grouping (day 0) for 14 days to control group and treated group, respectively. Tumor volume and body weight were measured two or three times a week for 42 days. [^{18}F]fluoro-2-deoxyglucose (^{18}F-FDG)-PET was carried out with an animal PET scanner (Inveon; Siemens, Knoxville, TN, USA) to assess the therapeutic effect of LAT1 inhibition, as ^{18}F-FDG-PET has been clinically used for therapeutic monitoring with regard to tumor metabolism. The ^{18}F-FDG was synthesized in our facility. Randomly selected mice were fasted for 12 h with free access to water before ^{18}F-FDG-PET. ^{18}F-FDG (10 MBq) was given i.v. to mice followed by 10 min of data acquisition at 2 h after the administration. Mice were maintained under isoflurane anesthesia through the administration of ^{18}F-FDG to PET scan. For analysis of the image, a region of interest was drawn around the edge of the tumor uptake of ^{18}F-FDG, and standardized uptake value (SUV) was calculated. The maximum value of SUV (SUVmax) in the region of interest was compared between BCH-treated mice and control mice.

Statistical analysis. Results are expressed as mean $\pm$ SEM. The statistical significance of differences between two groups was calculated using the unpaired Student's *t*-test. The statistical significance of differences between the control and other groups was calculated using Dunnett's test. The criterion of significance was $P < 0.05$, as determined with GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA).

Results

Expression profile of LAT and CD98 in human esophageal cancer cell lines. The expression of LAT1 mRNA was higher than other subtypes of LAT, and the expression of LAT2 mRNA was particularly low in both cell lines (Fig. 1a). The amount of LAT1 mRNA in KYSE150 cells was approximately 26-fold higher than that in KYSE30 cells. As shown in Figure 1(b), both LAT1 and CD98 protein were expressed in human esophageal cancer cells. Similar to mRNA expression, high expression of LAT1 protein was observed in KYSE150 cells. However, differences in CD98 expression were not observed.

L-leucine uptake and cell proliferation suppressed by BCH in esophageal cancer cells. The uptake of L-^{14}C-leucine, one of the substrates of LAT1,[24] was inhibited by treatment with BCH in a dose-dependent manner in both KYSE30 and KYSE150 cells (Fig. 2a). L-^{14}C-leucine uptake was higher in KYSE150 than in KYSE30 cells. The difference was consistent with the difference in the expression of LAT1. Cell proliferation was also suppressed by treatment with BCH in a dose-dependent manner (Fig. 2b). In contrast, the LDH level in culture medium was not different at 24 h after treatment with BCH, indicating that cell death was not induced by BCH treatment in esophageal cancer cells.

Cell cycle arrest and suppression of mTOR signaling induced by BCH. The mechanism of BCH-mediated growth inhibitory effect on the cell cycle and mTOR signaling were examined. The cell population in the G_0/G_1 phase was significantly increased at 24 and 48 h after treatment with 30 mM BCH in both KYSE30 and KYSE150 cells, indicating that BCH induced cell cycle arrest at G_1 phase (Table 1). Phosphorylation of mTOR was decreased at 30 min after treatment with BCH, but restored at the control level by 24 h in both KYSE30 and KYSE150 cells (Fig. 3). Phosphorylation of 4E-BP1 and p70S6K was decreased at 30 min and the decrease was continued for 24 h after treatment with BCH. The amount of mTOR, 4E-BP1, and p70S6K proteins was slightly decreased.

Antitumor activity of chemotherapeutic agents enhanced by BCH. The combined effect of BCH and chemotherapeutic agents on cell proliferation was examined. In this study, 5-FU and CDDP were used because they are standard chemotherapeutic agents for esophageal cancer. As shown in Figure 4, BCH decreased the survival fraction of both KYSE30 and KYSE150 cells treated with 5-FU and CDDP. These results indicate that BCH additively enhanced growth inhibition of these chemotherapeutic agents.

Systemic administration of BCH suppresses tumor growth in tumor-xenograft model. To investigate whether LAT1 inhibition could suppress tumor growth *in vivo*, BCH (200 mg/kg) was given i.v. to KYSE150-bearing mice. KYSE30 cells were inoculated into nude mice in the same manner as KYSE150 cells, but KYSE30-bearing mice could not be established because of excessive necrosis. As shown in Figure 5(a), daily treatment with BCH for 14 days significantly delayed tumor

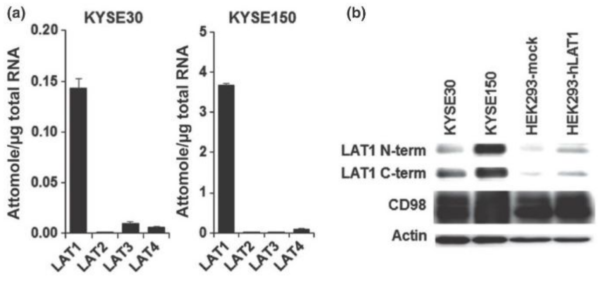

Fig. 1. Expression of system L amino acid transporters (LATs) and CD98 in KYSE30 and KYSE150 esophageal cancer cells. (a) Expression of LAT1, LAT2, LAT3, and LAT4 mRNA quantified by quantitative PCR ($n = 4$). Each quantity of LAT mRNA was calibrated by total RNA. (b) Protein expression of LAT1 and CD98 in KYSE30, KYSE150, HEK293-mock, and HEK293-hLAT1 cells. Actin was detected as the internal control. Representative images from three independent experiments were shown. C-term, C-terminal; N-term, N-terminal.

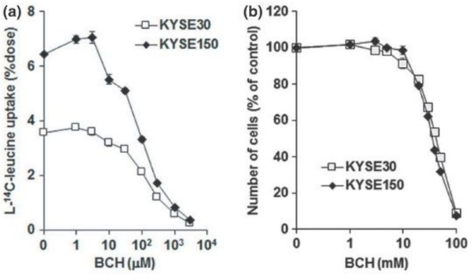

Fig. 2. 2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) inhibits leucine uptake and cellular growth. (a) BCH inhibits L-^{14}C-leucine uptake concentration-dependently in KYSE30 and KYSE150 esophageal cancer cells (n = 4). Ordinate shows a percentage of applied dose of L-^{14}C-leucine. (b) BCH inhibits the growth of KYSE30 and KYSE150 cells concentration-dependently (n = 4). Ordinate shows number of cells in a percentage of control (without BCH).

Table 1. Cell cycle profile in KYSE30 and KYSE150 esophageal carcinoma cells after treatment with 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) (n = 3)

KYSE30	G_0/G_1 ratio, %	S ratio, %	G_2/M ratio, %
24 h			
Control	28.82 ± 4.12	32.71 ± 1.88	34.36 ± 1.13
BCH (30 mM)	39.90 ± 3.52*	27.56 ± 4.48	28.52 ± 3.86
48 h			
Control	45.42 ± 2.00	25.80 ± 1.27	23.71 ± 0.85
BCH (30 mM)	55.18 ± 2.70**	18.90 ± 0.21***	19.53 ± 1.45*
KYSE150	**G_0/G_1 ratio, %**	**S ratio, %**	**G_2/M ratio, %**
24 h			
Control	42.21 ± 2.67	35.68 ± 2.00	23.06 ± 1.09
BCH (30 mM)	64.05 ± 1.31***	21.39 ± 0.84***	13.24 ± 0.77***
48 h			
Control	45.05 ± 0.97	30.30 ± 1.35	23.66 ± 0.63
BCH (30 mM)	62.82 ± 1.97***	21.62 ± 1.18**	13.64 ± 1.79***

*P < 0.05, **P < 0.01, ***P < 0.001, control versus BCH (30 mM).

growth. Significant decrease in the body weight as an indicator of toxicity was not observed in the BCH-treated mice (Fig. 5b). Body weight was decreased on day 2 and day 15 in both groups, which would be caused by 12 h of fasting for ^{18}F-FDG-PET. No animal died in this experiment. The SUVmax on ^{18}F-FDG-PET images on day 14 was increased in the control group, but decreased in the BCH-treated group, indicating that glucose metabolism of the tumor was decreased by treatment with BCH (Fig. 5c,d).

Discussion

The present study showed that LAT1 inhibition could induce antitumor effects in esophageal cancer. Selective LAT inhibitor BCH inhibited L-^{14}C-leucine uptake by the KYSE30 and KYSE150 cells, and then suppressed growth of esophageal cancer. It competitively inhibits the amino acid uptake by LAT, and has been commonly used as a LAT1 inhibitor in previous reports. The activity of amino acid transport of LAT1 is higher than in other subtypes of LAT,[3,25,26] and our results showed extremely high expression of LAT1 in both KYSE30 and KYSE150 cells. Thus, these results indicate that BCH could inhibit LAT1-dependent amino acid uptake in these esophageal cancer cells. It is clear that LAT1 is significantly related to survival and growth of cancer through amino acid supply and signal regulation. Antitumor efficacy of LAT1 inhibition has been determined in various types of cancer.[6,12,16–19] Therefore, LAT1 is an attractive target for molecular targeting therapy.

The amounts of LAT1 protein in KYSE30 and KYSE150 cells were remarkably different, whereas the amount of CD98 protein was similar. The expression of LAT1 shown by immunoblotting was the amount of LAT1 protein in whole cell lysate. As LAT1 is expressed on the cell membrane after heterodimerization with CD98, the amounts of functional LAT1 are regulated by the expression of CD98.[14] The expression of CD98 and LAT1 in KYSE cells was semiquantified by densitometry, and the ratios of each protein (KYSE150/KYSE30) were 1.68 ± 0.42 (CD98, n = 3), 5.22 ± 1.04 (LAT1 N-terminal, n = 3), and 6.00 ± 2.30 (LAT1 C-terminal, n = 3). The ratio of L-^{14}C-leucine uptake between KYSE30 and KYSE150 cells was approximately 1.81 (KYSE150/KYSE30), which represents the ratio of functional activity of LAT1 on the cell membrane. The ratio of L-^{14}C-leucine uptake was close to the ratio of CD98. These results strongly support the formation of heterodimerization of LAT1 with CD98 in esophageal cancer cells, and indicate that the amounts of LAT1 on cell membrane was close between KYSE30 and KYSE150. Therefore, remarkable differences would not be observed in growth inhibition or mTOR signaling.

The cellular nutrient condition critically affects the activity of mTOR. In amino acid-rich conditions, rapamycin and nutrient-sensitive complex 1 (mTORC1) is recruited to the surface of lysosomes and activated by the vacuolar H$^+$-ATPase-Regulator-Rag complex.[27,28] Activated mTORC1 phosphorylates its downstream molecules, p70S6K and 4E-BP1, which are deeply associated with protein synthesis.[15] However, amino

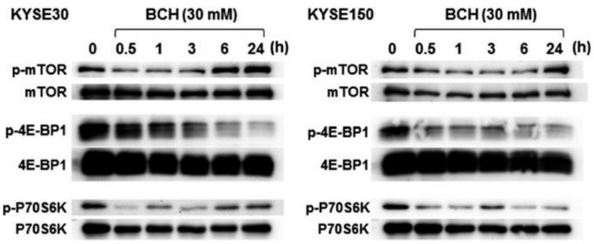

Fig. 3. 2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) blocked the phosphorylation (p) of mammalian target of rapamycin (mTOR), p70S6K, and 4E-BP1 in KYSE30 and KYSE150 esophageal cancer cells. Whole proteins of mTOR, p70S6K, and 4E-BP1 were detected as control. Representatives from three independent experiments were shown.

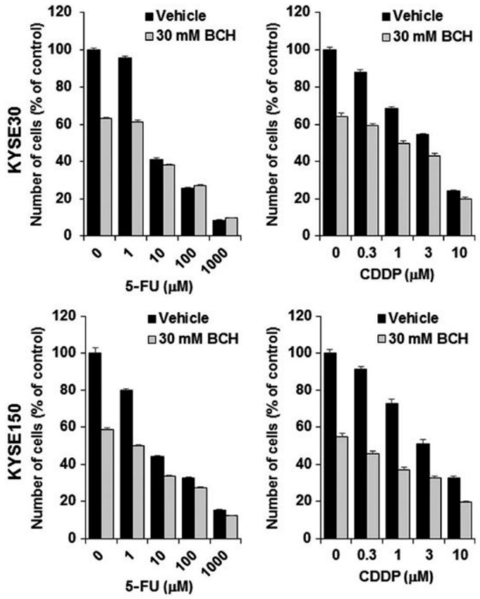

Fig. 4. 2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) treatment enhances antitumor effect of 5-fluorouracil (5-FU) and cisplatin (CDDP) on KYSE30 and KYSE150 esophageal carcinoma cells. Ordinate shows number of cells in a percentage of control ($n = 4$).

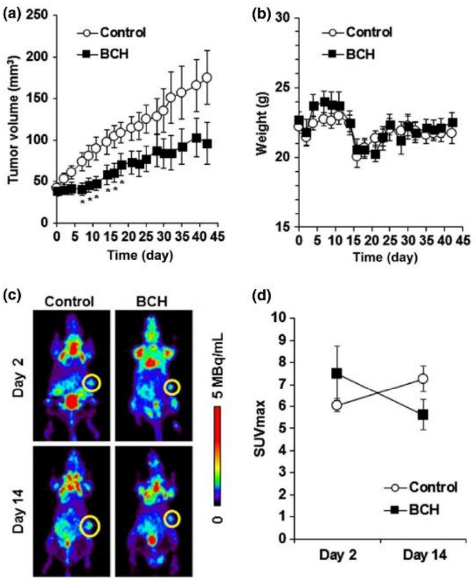

Fig. 5. Antitumor effect of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) on KYSE150 tumor-xenograft model. (a) Growth curves of KYSE150 tumor after treatment with saline or BCH ($n = 10$). *Statistically significant difference from control ($P < 0.05$). (b) Body weight of KYSE150 tumor-bearing mice after treatment with saline or BCH ($n = 10$). (c) Representative coronal section of ^{18}F-FDG-PET images of KYSE150-bearing mice at 2 h after ^{18}F-FDG injection. PET imaging was carried out at indicated days after the day of grouping. Yellow circle shows the tumor. The calibration bar is shown to the right of images. (d) Maximum standardized uptake value (SUVmax) of ^{18}F-FDG-PET images at day 2 and day 14 after the day of grouping ($n = 4$).

acid deprivation inactivates mTORC1. The present study revealed that BCH suppressed phosphorylation of mTOR, 4E-BP1, and p70S6K in esophageal cancer cells. As LAT1 mediates transport of essential amino acids, such as L-leucine, L-phenylalanine, and L-tryptophan,[3] LAT1 inhibition with BCH would lead to deprivation of intracellular essential amino acids, followed by suppression of mTOR signaling. Inhibition of LAT1 with BCH might be involved in the slight decrease of mTOR, 4E-BP1, and p70S6K proteins, which would also contribute to the suppression of phosphorylation of these proteins. In amino acid-deprived conditions, cells degrade intracellular components and maintain intracellular amino acid levels for essential cellular functions; this process is called autophagy.[29] In our study, phosphorylation of these proteins had been resumed by 24 h after BCH treatment. This might be caused by recycling of amino acids induced by autophagy.[30] The mTOR signal also has critical roles in cell cycle progression. It has been reported that suppression of mTOR leads to G_1 arrest and both p70S6K and 4E-BP1 independently mediate mTOR-dependent G_1 phase progression.[31] Therefore, it is suggested that LAT1 inhibition with BCH suppressed mTOR signaling, followed by G_1 cell cycle arrest and suppression of cell proliferation in esophageal cancer. Kim *et al.*[16] reported that LAT1 inhibition with BCH induced apoptosis together with cell cycle arrest in oral cancer cells. In this study, BCH significantly inhibited cell growth but did not induce cell death, suggesting that LAT1 inhibition induces a cytostatic effect in esophageal cancer cells. Inhibitory effects of LAT1 inhibition would differ between the types of cancer.

Growth inhibition with conventional chemotherapeutic agents has been enhanced by LAT1 inhibition in several types of cancer.[12,19,32,33] In our study, BCH additively enhanced the growth-inhibitory effect of 5-FU and CDDP in esophageal cancer cells. Both 5-FU and CDDP induce DNA damage, and BCH inhibits amino acids supply and mTOR signaling. Simultaneous inhibition of different growth processes would cause additive antitumor efficacy. In tumor-bearing mice, daily injection of BCH significantly delayed tumor growth. The decrease of ^{18}F-FDG accumulation in tumor also supports the growth suppression induced by BCH treatment *in vivo*. Antitumor effects of LAT1 inhibition have been well investigated *in vitro*, but there have been few reports showing antitumor effects with LAT1 inhibition *in vivo*. Thus, our results strongly suggest that LAT1 inhibition could be a novel molecular target for esophageal cancer therapy. As the therapeutic effect of molecular-targeting drugs, such as cetuximab and bevacizumab, to esophageal cancer have not been observed in the present clinical studies, LAT1 inhibition would have a great impact as a novel therapeutic target. Although side-effects were not observed after BCH treatment, it would be difficult to use BCH itself as an antitumor agent in esophageal cancer therapy, because BCH is not a selective inhibitor of LAT1 and its antitumor effect was limited. Oda *et al.*[17] developed the LAT1-selective inhibitor, KYT-0353, and reported its remarkable antitumor activity in colon cancer-bearing mice. Furthermore, chemical conformation for more potent and selective inhibition to LAT1 has recently been reported.[34] Selective inhibitor of LAT1, like KYT-0353, would increase efficacy and decrease adverse events in patients with esophageal cancer.

In this study, two types of esophageal cancer cell line were used; KYSE30 is a well-differentiated SCC, and KYSE150 is a poorly differentiated SCC.[21] Among them, the expression level of LAT1 was markedly higher in KYSE150 cells. Many reports have shown that the high expression of LAT1 could be related to poor prognosis in various types of cancer.[7–13] Also, the prognosis of patients with poorly differentiated cancer is generally dismal. It has been shown that the expression of LAT1 was higher in well-differentiated cancer compared to poorly differentiated cancer.[35,36] Although the relationship between LAT1 expression and cancer differentiation is still unclear, there might be a novel clinical implication of LAT1 inhibition therapy and cancer differentiation.

In conclusion, LAT1 inhibition with BCH suppressed leucine uptake, mTOR signaling, cell cycle progression, and cell proliferation in esophageal cancer cells and significantly delayed tumor growth in tumor-bearing mice. Furthermore, LAT1 inhibition could also enhance the antitumor effects of conventional chemotherapeutic drugs in esophageal cancer. Therefore, LAT1 could be a novel promising target for esophageal cancer therapy.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Primers for the synthesis of full-length system L amino acid transporters (LATs) and quantitative PCR.

Table S2. Antibodies used in immunoblotting.