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Translational Relevance

Chemotherapy is one of the essential treatments in esophageal cancer. It is also important to predict the response to chemotherapy before treatment to avoid unnecessary treatment. In this study, we investigated whether we could predict the response to cisplatin-based chemotherapy for esophageal cancer by analyzing the miRNA expression using biopsy samples before treatment. Of the several miRNAs associated with resistance to cisplatin, let-7b and let-7c expression is potentially useful to predict the response to chemotherapy. We also found that let-7 modulates the chemosensitivity to cisplatin through the regulation of interleukin (IL)-6/STAT3 pathway in esophageal cancer. This result should help doctors and scientists dealing with chemotherapy for gastrointestinal cancers including esophageal cancer.

of upregulated miR-200c expression in chemoresistance in esophageal cancer and that this effect is mediated through activation of the Akt signaling pathway (14).

In the present study, we examined whether we could predict the response to chemotherapy before treatment in patients with esophageal cancer, by using endoscopic biopsies. The results showed that low expression of let-7 measured before treatment is associated with low sensitivity to cisplatin-based chemotherapy in esophageal cancer. The molecular mechanism of the involvement of let-7 expression in chemosensitivity was also investigated.

Materials and Methods

Patients, treatment, and samples

Biopsy samples were obtained under esophagoscopy from 98 patients with histopathologically confirmed primary thoracic esophageal squamous cell carcinoma who subsequently underwent surgical resection between 2000 and 2011 at the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan. Informed consent was obtained from each patient before participation in this study. These 98 patients were divided at random into 2 independent groups; 74 in the training set and the remaining 24 patients in the validation set. Biopsy samples of the patients were obtained before preoperative chemotherapy. The samples were confirmed to contain cancerous tissue. All patients received neoadjuvant chemotherapy, which consisted of 2 courses of 5-fluorouracil (5-FU), cisplatin, and Adriamycin, using the following protocol: Cisplatin was administered at 70 mg/m^2, Adriamycin was administered at 35 mg/m^2 intravenously on day 1, and 5-FU was administered continuously from days 1 to 7 at 700 mg/m^2/d. Two courses of chemotherapy were provided after an interval of 4 weeks (8). The median follow-up period was 22.4 months. Thirty (30.6%) patients died during the follow-up period. Patients were divided into 2 groups: the first 74 patients were categorized as the training set whereas the second group of 24 patients was categorized as the validation set (Supplementary Table S1).

Clinical and histopathological evaluation of response to chemotherapy

The clinical response to chemotherapy was evaluated according to the World Health Organization Response Criteria for Measurable Diseases (15). Complete response (CR) represented total regression of the tumor. Partial response (PR) represented more than 50% reduction in primary tumor size on computed tomography (CT). Progressive disease (PD) represented more than 25% increase in the primary tumor or appearance of new lesion. Stable disease (SD) represented cases that did not meet the criteria of PR or PD. For evaluation, both the CR and PR were grouped together into the responders whereas the SD and PD were grouped as non-responders. The clinical response was assessed retrospectively by 2 investigators (K. Sugimura and H. Miyata) in a blinded fashion. The histopathologic response was also categorized according to the criteria of the Japanese Society for Esophageal Diseases (16). The percentage of viable residual tumor cells within the entire cancerous tissue was defined as follows: grade III, no viable residual tumor cells; grade II, less than two-third residual tumor cells; grade I, more than two-third residual tumor cells; and grade 0, no significant response to chemotherapy. The histopathologic response was assessed retrospectively by 2 investigators (K. Sugimura and K. Tanaka) in an independent manner and any disagreements were resolved by consensus.

Cell culture

Human esophageal squamous cell lines, TE1/TE5/TE8/TE9/TE10/TE11/TE13, were obtained from the Riken Bioresource Center Cell Bank. All cells were cultured in RPMI-1640 media (Life Technologies), containing 10% FBS (Sigma-Aldrich Co.) and 1% penicillin/streptomycin (Life Technologies), in a humidified atmosphere under 5% CO_2 at 37°C.

Establishment of cisplatin-resistant cell lines

Cisplatin-resistant cell lines (TE8-R and TE10-R) were cultured through gradual increase in cisplatin concentration [*cis*-diamminedichloroplatinum (II), Wako], as described previously (14). The cultured cells were exposed cisplatin at an initial concentration of 2 μmol/L. Three days later, the cells were cultured in cisplatin-free medium until confluence. Next, cisplatin concentration was increased by 2- to 3-fold. This cycle was repeated until cisplatin concentration reached 35 μmol/L.

Isolation of RNA

Total RNA was isolated from cells or tissues using TRIzol reagent (Life Technologies) according to the protocol provided by the manufacturer. Briefly, 100 mg of tissue samples was homogenized with 1 mL of TRIzol reagent using a power homogenizer. After homogenization, the samples were mixed with 0.2 mL of chloroform. The samples were shaken vigorously for 15 seconds and then centrifuged at 12,000 × *g* for 15 minutes at 4°C. The supernatant in the tube was mixed with 0.5 mL of 100% isopropanol and then incubated at room temperature for 10 minutes. After centrifugation at 12,000 × *g* for 10 minutes at 4°C, the supernatant was removed and washed with 1 mL of 75% ethanol. After centrifugation at 7,500 × *g* for 5 minutes at 4°C, the supernatant was removed and the pellet was dried for 5 minutes. The RNA pellet was resuspended in RNase-free water and adjusted into appropriate concentration.

Reverse transcription PCR and TaqMan miRNA assay

TaqMan miRNA Assay (Applied Biosystems) was used to measure miRNA levels. This assay detects only the mature form of the specific miRNAs. First, 10 ng of RNA was reverse transcribed and the resulting cDNA was amplified using the following specific TaqMan microRNA assays. Assay IDs were hsa-miR-135a ID 000460, hsa-miR-96 ID 000186, hsa-miR-141 ID 000463, hsa-miR-101 ID 2253, hsa-miR-146a ID 000468, hsa-miR-489 ID 0002358, hsa-miR-545 ID 0002267, hsa-miR-99a ID000435, hsa-let-7b ID 002619, hsa-miR-204 ID 000508, hsa-let-7c ID 000379, hsa-miR-202 ID 002363, hsa-miR-10a ID 000387, hsa-miR-136 ID 000592, hsa-miR-145 ID 002278, and RNU48 ID:001006. The PCRs were carried out in the 7500HT Sequence Detection System (Applied Biosystems), as recommended by the manufacturer. Amplification data were normalized to RNU48 expression. Quantification of relative expression was conducted using the $2^{-\Delta\Delta C_t}$ method (17).

Interleukin-6 quantitative reverse transcription PCR

For reverse transcriptase reaction, the Reverse Transcription System (Promega) was used according to the protocol provided by the manufacturer. Real-time quantitative reverse transcription PCR (qRT-PCR) was carried out using designed oligonucleotide primers and Light Cycler (Roche Diagnostics), and the amount of interleukin (IL)-6 mRNA expression was calculated. The expression of IL-6 was normalized relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control. The designed PCR primers were as follows: IL-6: forward primer, 5′-CCTTCCAAAGATGGCTGAAA-3′, reverse primer, 5′-ATCTGAGGTGCCCATGCTAC-3′; GAPDH: forward primer, 5′-CAACTACATGGTTTACATGTTC-3′, reverse primer, 5′-AAATGAGCCCCAGCCTTC-3′.

miRNA microarray

The miRNA expression profiling was conducted with 1,000 ng of RNA extracted from 2 esophageal cell lines (TE8 and TE10) and the corresponding cisplatin-resistant cell lines (TE8-R and TE10-R) using the TaqMan Array Human MicroRNA Panel (version 1, Applied Biosystems). This qRT-PCR array contains the 365 target microRNAs as well as the endogenous controls. Normalization was conducted with RNU48. The expression of each miRNA in cisplatin-resistance cell line was compared with that in the control parent cell line, and the ratio of miRNA expression in cisplatin-resistance cell line to control cell line was calculated for all 365 miRNAs.

miRNA transfection

TE11 and TE13 cells were transfected with 30 nmol/L pre-miR miRNA precursor molecules of has-let-7c (#PM10436, Applied Biosystems) using SiPORT NeoFX (Ambion) in 6-well plates or 6-cm dishes according to the instructions supplied by the manufacturer. Pre-miR negative control (Applied Biosystems) was also used as a control.

MTT assay

Cell viability was determined by MTT (Sigma-Aldrich) assay. Let-7c or negative control miRNA–transfected cells were seeded into 96-well plates in culture medium. After 24 hours, the medium was changed with a medium containing the following concentration of cisplatin (0, 3.125, 6.25, 12.5, 25, 50, 100, 200, or 400 μmol/L). After incubation for 6 hours, the medium was changed into normal medium. Seventy-two hours after culture, the cells were stained with 20 μL MTT (5 mg/mL) at 37°C for 4 hours and subsequently solubilized in 100 μL of 0.004N HCl-isopropanpol. Absorbance was measured at 490 nm using a microplate reader (Bio-Rad Laboratories).

Apoptosis assay

Apoptosis was assessed by the flow cytometric detection of phosphatidyl serine externalization using Annexin V and propidium iodide staining (Bio Vision). TE13 cells, after transfection with pre-let-7c and pre-miR negative controls, were treated with 40 μmol/L cisplatin for 6 hours. The cells were harvested and processed for Annexin V staining using the procedure described by the supplier. Briefly, cells were trypsinized gently and resuspended with 500 μL of 1× binding buffer and then treated with 5 μL of Annexin V-FITC and 5 μL of phosphatidylinositol (PI). After incubation for 5 minutes on ice, each sample was analyzed immediately using the FACSCalibur flow cytometer (BD Bioscience).

ELISA assay

After 24-hours culture, the cells were exposed to 5 μmol/L CDDP (mentioned above) or medium only. The supernatants were collected (24, 48, or 72 hours) and centrifuged. IL-6 protein level was measured using ELISA kits (#D6050, R&D Systems) according to the protocol provided by the manufacturer.

Western blotting

Cells were washed with ice-cold PBS and harvested from the culture dish. The cells were lysed in RIPA buffer (25 mmol/L Tris, pH 7.5, 50 mmol/L NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, and 500 KIE/mL aprotinin) containing phosphatase inhibitor. The extracts were centrifuged and the supernatant fractions were collected for Western blot analysis. The following antibodies were used

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in this study: at 1:2,000 for anti-human p-STAT3 (Tyr705) antibody (#9145, Cell Signaling), 1:2,000 for anti-human STAT3 antibody (#9132, Cell Signaling), 1:2,000 for anti-human p-Akt antibody (#9271, Cell Signaling), 1:2,000 for anti-human Akt antibody (#4691, Cell Signaling), 1:2,000 for anti-human Erk antibody (#4370, Cell Signaling), 1:2,000 for anti-human Erk antibody (#4695, Cell Signaling), 1:10,000 for anti-human β-actin (#A2066, Sigma-Aldrich), and 1:2,000 for all secondary antibodies. Immune complexes were detected using the Detection Kit (GE HealthCare).

Statistical analysis

To validate the clinical significance of let-7c expression as a marker of chemosensitivity in patients with esophageal cancer, we used the cross-validation method. Data were expressed as mean ± SD. Clinicopathologic parameters were compared using the χ^2 test and continuous variables were compared using Student t test. Survival curves were computed using the Kaplan–Meier method, and differences between survival curves were compared using the log-rank test. $P < 0.05$ denoted the presence of a statistically significant difference. Statistical analysis was conducted using the JMP Ver. 8.0 software.

Table 1. Fold change in the expression of 15 microRNAs in cisplatin-resistant cells compared with parental cells

microRNA	TE8R/TE8 fold change	TE10R/TE10 fold change
Upregulation		
miR-135a	6.08	12.07
miR-96	3.40	3.85
miR-141	2.41	25.37
miR-101	1.75	2.21
miR-146a	1.97	1,556.1
miR-489	1.78	5.30
miR-545	1.84	3.09
Downregulation		
miR-99a	0.49	0.12
let-7b	0.37	0.39
miR-204	0.35	0.29
let-7c	0.26	0.11
miR-202	0.02	0.01
miR-10a	0.57	0.06
miR-145	0.52	0.03
miR-136	0.54	0.002

Results

Altered expression of 15 miRNAs in cisplatin-resistant cells

PCR-based microarray analysis was conducted to compare the expression of miRNAs in cisplatin resistance cells and control cells using 2 pairs of cell lines; TE8/TE8-R and TE10/TE10-R. The miRNA microarray analysis in TE8/TE8-R and TE10/TE10-R cisplatin-resistant cells showed altered expression (by more than 1.7-fold) in 128 (35.0%) and 177 (48.5%) miRNAs among 365 miRNAs, respectively, compared with control cells. Among the miRNAs with altered expression in cisplatin-resistant cells, 15 miRNAs showed overlap in the 2 cell lines. Among these 15 miRNAs, miR135a, miR-96, miR-141, miR-101, miR-146a, miR-489, and miR-545 were upregulated, whereas miR-99a, let-7b, miR-204, let-7c, miR-202, miR-10a, miR-136, and miR-145 were downregulated in cisplatin-resistant cells, compared with control cells (Table 1). Accordingly, we selected these 15 miRNAs as candidates for the response to chemotherapy in esophageal cancer.

Low expression of let-7c is associated with poor response to chemotherapy and poor prognosis

To determine whether the 15 miRNAs are implicated in the response to chemotherapy, we carried out qRT-PCR using pretreatment biopsy samples in 74 patients in training set group with esophageal cancer who underwent preoperative chemotherapy followed by surgery (Table 2). With regard to the clinical response in 74 patients of the training set, CR and PR was achieved in 3 and 30 patients, respectively, whereas SD and PD was observed in 35 and 6 patients, respectively. Thus, 33 (44.6%) patients were categorized as responder whereas the remaining 41 (55.4%) patients were categorized as nonresponders. Expression of the 15 miRNAs was confirmed in the biopsy samples. We also divided the 74 patients of the training set into 2 groups on the basis of the median value of the expression level of each miRNA; the high expression group ($n = 37$) and the low expression group ($n = 37$). Among 15 selected miRNAs, high expression levels of let-7b and let-7c correlated significantly with the clinical response to chemotherapy in esophageal cancer ($P = 0.019$, $P = 0.005$ respectively). However, the expression of the other microRNAs did not correlate with chemosensitivity. Next, we examined whether the expression of let-7b and let-7c is associated with the histopathologic response. With regard to the histopathologic response in 74 patients of the training set, complete tumor regression (grade III) and major tumor regression (grade II) was observed in 3 and 9 patients, respectively, whereas minor tumor regression (grade I) and almost no tumor regression (grade 0) was observed in 54 and 8 patients, respectively. Similar to the clinical response, high expression of let-7b and let-7c correlated significantly with better histopathologic response (Fig. 1A and B). Thus, the expression of let-7b and let-7c in pretreatment biopsy samples determined the response to chemotherapy in patients with esophageal cancer.

Next, we examined whether the expression of let-7b and let-7c is associated with the prognosis of patients who underwent preoperative chemotherapy followed by surgery for esophageal cancer. High expression of let-7c correlated significantly with longer survival in patients who received preoperative chemotherapy (Fig. 1D). High expression of

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Table 2. Relationship between the expression of 15 microRNAs and clinical response

miRNA	Responders ($n = 33$) high/low	Nonresponders ($n = 41$) high/low	*P*
miR-135a	23/10	14/27	0.640
miR-96	19/14	18/23	0.350
miR-141	19/14	18/23	0.350
miR-101	19/14	18/23	0.350
miR-146a	20/13	17/24	0.160
miR-489	18/15	19/22	0.640
miR-545	19/14	18/23	0.350
miR-99a	15/18	22/19	0.640
let-7b	22/11	15/26	0.019
miR-204	15/18	22/19	0.640
let-7c	23/10	14/27	0.005
miR-202	16/17	21/20	1.000
miR-10a	21/12	16/25	0.061
miR-145	20/13	17/24	0.160
miR-136	16/17	21/20	1.000

NOTE: Data are number of patients.

let-7b also tended to correlate with longer survival, but this tendency did not reach statistical significance (Fig. 1C). We could not find significant relationship between let-7c expression and any clinicopathologic parameter in patients who received preoperative chemotherapy followed by surgery.

To validate the clinical significance of let-7c expression as a marker of chemosensitivity in patients with esophageal cancer, we examined the relationship between let-7c expression and chemosensitivity using biopsy samples of the second group of 24 patients in validation set group. The results confirmed that high expression of let-7c also correlated significantly with the clinical response in esophageal cancer.

Induction of let-7c expression restores chemosensitivity and increases apoptosis after genotoxic chemotherapy

In the next series of studies, we established the relationship between let-7c expression and chemosensitivity using esophageal squamous cell carcinoma cell lines. First, we determined let-7c expression in each esophageal cancer cell line and found relatively low expression of let-7c in TE11 and TE13 cells compared with other esophageal cancer cell lines (Supplementary Fig. S1a). To evaluate the biologic effect of let-7c, pre-let-7c was transfected into TE11 and TE13 cells, and let-7c expression was confirmed in the let-7c–transfected cells (Supplementary Fig. S1b). The MTT assay showed that let-7c–transfected cells were significantly more sensitive to cisplatin than control cells. Furthermore, the IC_{50} of let-7c–transfected

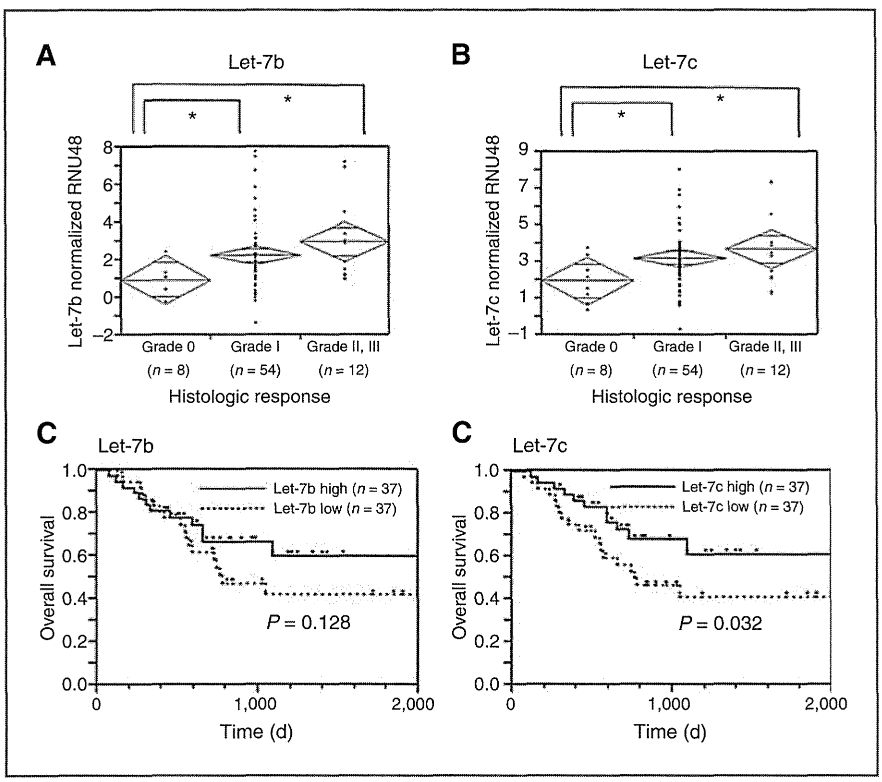

Figure 1. Association of let-7b and let-7c expression with histologic response and overall survival of patients treated with preoperative chemotherapy. A and B, the expression of let-7b and let-7c was higher in patients with histologic response of grade II–III/I than in those with grade 0 (let-7b: $P = 0.014/0.02$; let-7c: $P = 0.032/0.025$). C and D, overall survival curves of 74 patients with esophageal cancer according to let-7b and let-7c expression. High expression of let-7c correlated significantly with longer survival ($P = 0.032$). High expression of let-7b showed similar effect ($P = 0.128$).

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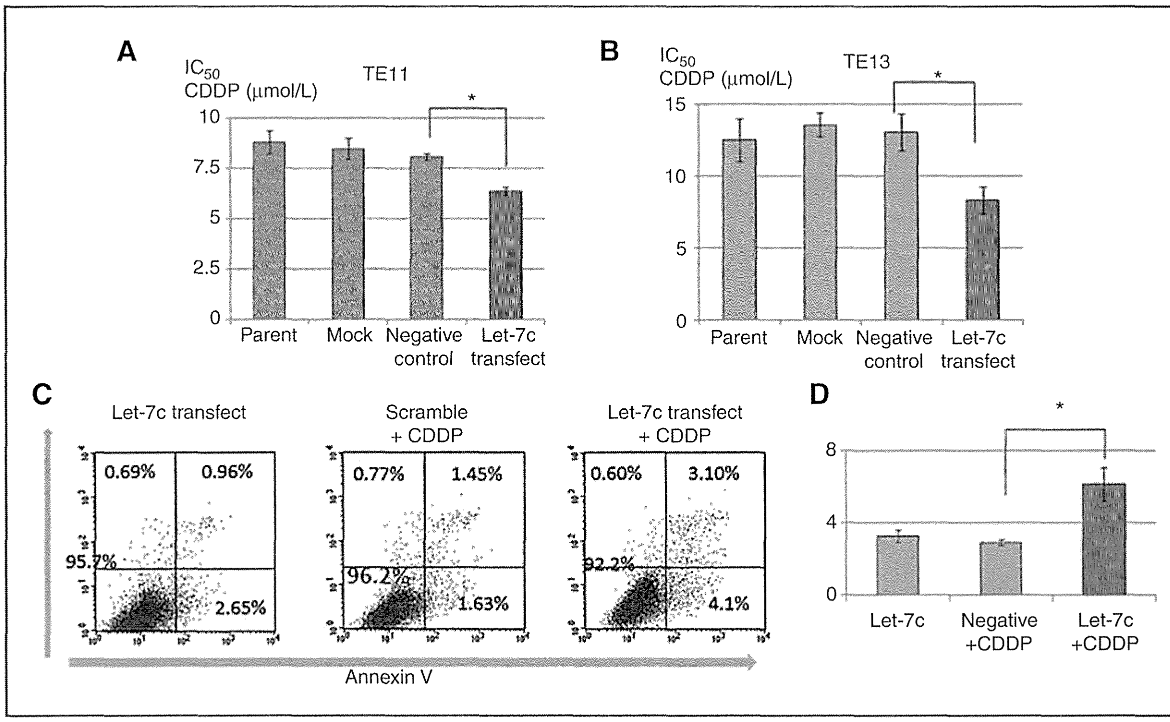

Figure 2. Overexpression of let-7c increases chemosensitivity in esophageal cells. A and B, the IC_{50} levels of cisplatin in TE11 and TE13 esophageal cells were significantly lower in let-7c transfected cells than in negative control transfected cells. Data are mean ± SD. *, $P < 0.01$. C, apoptotic cells were detected by flow cytometry using Annexin V and PI staining. Apoptotic cells were regarded as Annexin-V–positive cells. D, transfection of let-7c significantly increased the proportion of apoptotic cells after cisplatin treatment, compared with the negative control. Data are mean ± SD of 3 experiments. *, $P < 0.01$.

cells was significantly smaller than that of the negative control (Fig. 2A and B).

We also examined the effect of let-7c transfection on apoptosis. For this purpose, we used flow cytometry to determine the percentages of Annexin-V–positive cells among let-7c–transfected cells and control cells treated with cisplatin. Transfection of let-7c significantly increased the proportion of apoptotic cells after cisplatin treatment, compared with the negative control (2.9% vs. 6.1% at 24 hours, $P = 0.049$, Fig. 2C and D). Thus, induced expression of let-7c restored chemosensitivity and increased apoptosis after genotoxic chemotherapy in esophageal cancer cells.

Cisplatin activates IL-6/STAT3 prosurvival signaling pathway

What is the mechanism of let-7c–mediated chemosensitivity of esophageal cells? To answer this question, we hypothesized that let-7c expression regulated apoptosis in cisplatin-treated cells through downregulation of IL-6–mediated signaling pathway. This was based on Target scan and miRBase Targets database, which showed that IL-6 is potential target of let-7c, and also on previous finding of IL-6 as a putative let-7 target (18). In addition, a recent study has shown that IL-6 is released by genotoxic chemotherapy to protect cancer cell from cell death (19). First, we showed that cisplatin activated IL-6 mRNA in esophageal cancer cells (Fig. 3A). Next, we assayed IL-6 levels by ELISA. Cisplatin significantly increased the amount of IL-6 in the conditioned media (Fig. 3B). Furthermore, phosphorylated STAT3, which is downstream of IL-6, was induced by cisplatin in esophageal cancer cells (Fig. 3C and D). These results suggest that cisplatin activates the IL-6/STAT3 signaling pathway in an autocrine manner in esophageal cancer cells.

Next, we investigated whether activation of IL-6/STAT3 pathway protects cisplatin-exposed cancer cells from apoptosis. For this purpose, we examined cell viability and apoptosis in cisplatin-treated IL-6 knockdown cells and control cells. MTT assay showed that knockdown of IL-6 in esophageal cancer cells significantly reduced cell viability (Fig. 3E), and Annexin V assay showed that knockdown of IL-6 in esophageal cancer cells significantly increased the rate of apoptosis (Fig. 3F and G). These results indicate that cisplatin activates IL-6/STAT3 pathway in cancer cells, paradoxically providing protection of cancer cells against cell death.

Let-7 represses IL-6/STAT3 prosurvival pathway after genotoxic chemotherapy

We examined whether let-7 represses the activation of IL-6/STAT3 signaling pathway after cisplatin chemotherapy. Expression of IL-6 mRNA was significantly reduced after

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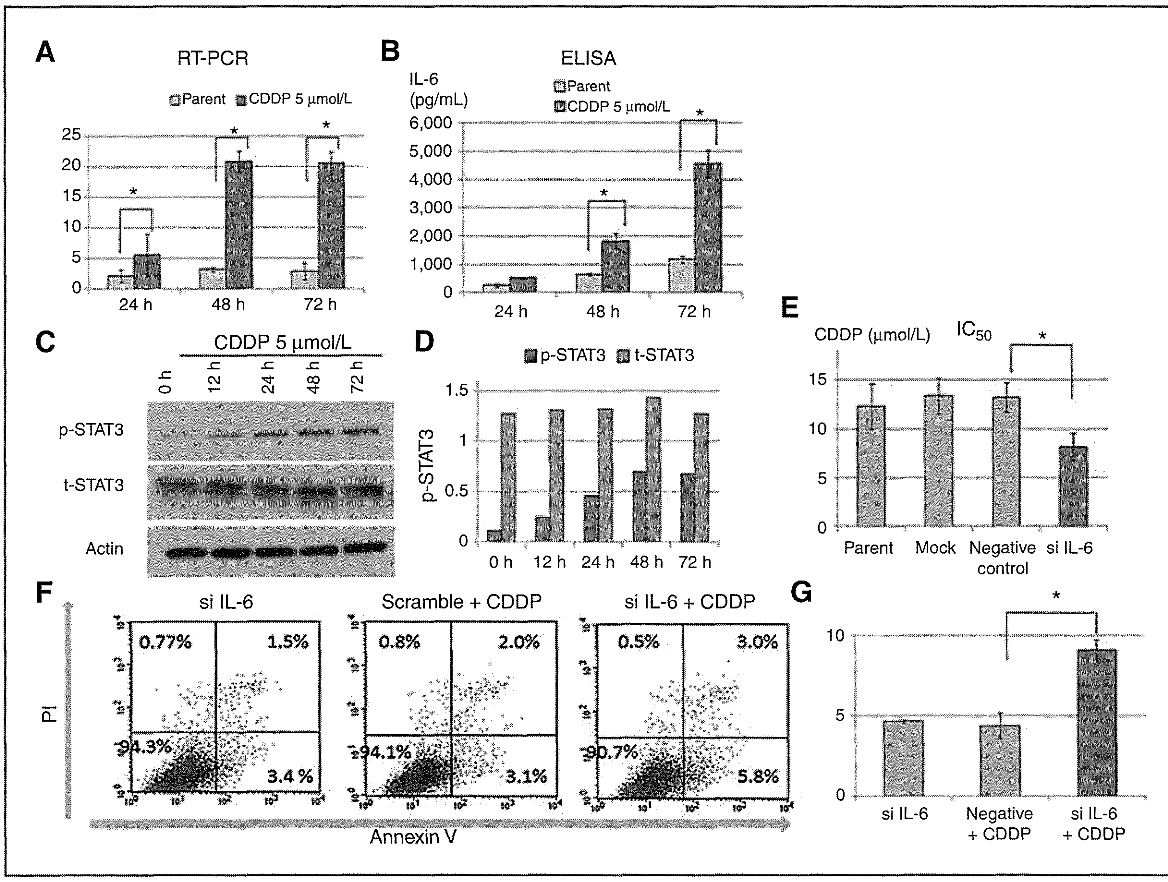

Figure 3. Cisplatin activates prosurvival IL-6/STAT3 signaling pathway. A, cisplatin significantly increased the expression of IL-6 mRNA in esophageal cancer cells at 24-, 48-, and 72-hour exposure. B, cisplatin significantly increased the expression of IL-6 protein in supernatants of conditioned medium at 24-, 48-, and 72-hour exposure. C, Western blot analysis of phosphorylated STAT3 and total STAT3 after cisplatin exposure. Exposure to cisplatin induced phosphorylated STAT3 in esophageal cancer cells. p-STAT3, phosphorylated STAT3; t-STAT3, total STAT3. D, semiquantitative analyses of expression of pSTAT3 and t-STAT3 in (C) by using densitometer. E, the IC_{50} level of cisplatin in siIL-6–transfected cells is significantly lower than in negative control transfected cells. F and G, transfection of siIL-6 significantly increased the proportion of apoptotic cells after cisplatin, compared with the negative control. Data in (A), (B), (E), and (G) are mean ± SD of 3 experiments. *, $P < 0.01$.

cisplatin treatment in let-7c transfected cells compared with control cells. The level of secreted IL-6 in the conditioned medium after cisplatin treatment was also significantly reduced in let-7c–transfected cells compared with control cells (Fig. 4A). Furthermore, phosphorylated STAT3 was significantly reduced in let-7c–transfected cells compared with control cells after cisplatin treatment, although the induced expression of let-7c had no apparent effect on the expression of Akt and extracellular signal–regulated kinase (Erk), which are downstream of IL-6 (Fig. 4B and C). Taken together, these results indicate that let-7 represses IL-6/STAT3 prosurvival pathway after genotoxic chemotherapy in esophageal cancer cells.

Finally, we examined the relationship between let-7c and IL-6 expression in clinical samples obtained from 40 patients with esophageal cancer. Let-7c expression of cancer tissue is significantly lower than that of noncancerous tissue (Fig. 4D). In contrast, IL-6 expression was significantly higher in cancer tissue than in noncancerous tissue (Fig. 4E). Moreover, IL-6 expression correlated inversely with let-7c expression in noncancerous tissue and esophageal cancer tissue (Fig. 4F).

Discussion

In multimodal therapy for esophageal cancer, chemotherapy is often combined with radiation and/or surgery. If prediction of the response to chemotherapy before surgery is possible, one can offer another treatment option for patients who show resistance to chemotherapy. In the present study, we investigated whether we could predict the response to cisplatin-based chemotherapy by analyzing the miRNA expression in esophageal cancer using biopsy samples before treatment. The results showed that low expression of let-7b and let7c is associated with low chemosensitivity in patients with esophageal cancer. The

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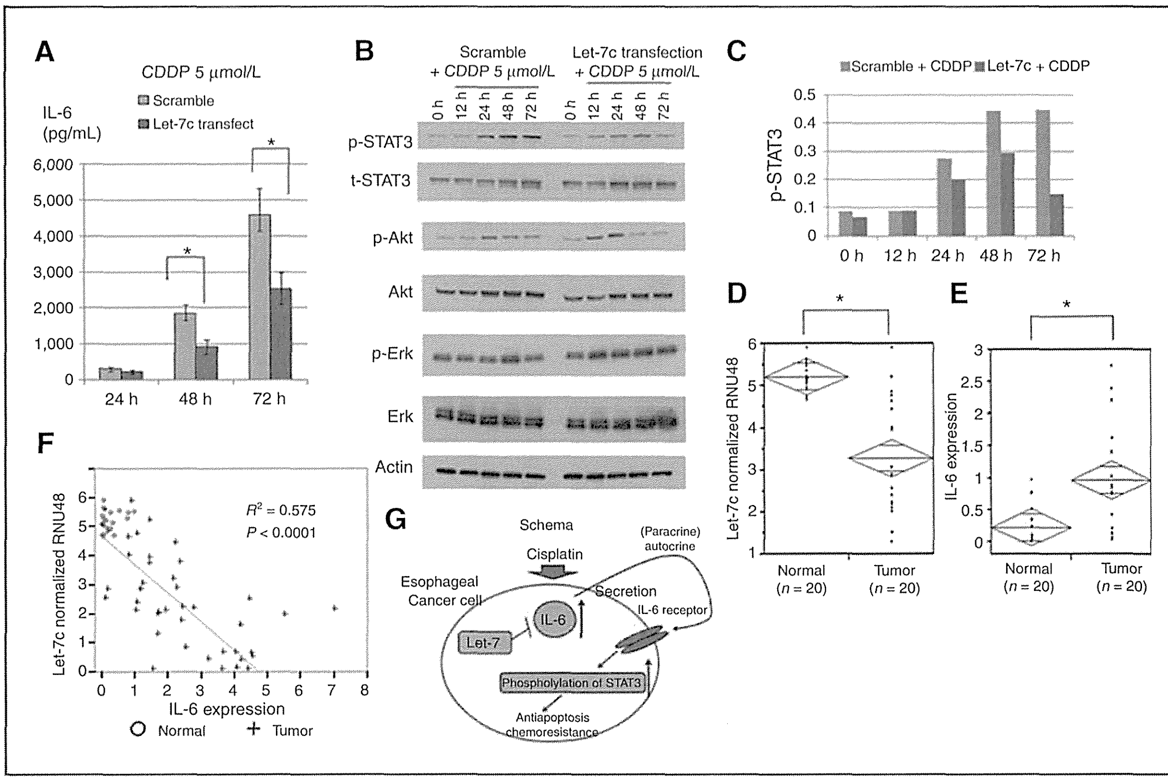

Figure 4. Overexpression of let-7c represses IL-6/STAT3 prosurvival pathway after cisplatin exposure. A, cisplatin significantly reduced IL-6 production in conditioned medium of let-7c–transfected cells compared with control cells at 48 and 72 hours. Data are mean ± SD of 3 experiments. *, $P < 0.01$. B, Western blot analysis of differential expression of proteins downstream of IL-6 after cisplatin exposure. Phosphorylated STAT3 was significantly reduced in let-7c–transfected cells compared with control cells. Representative data of 3 experiments with similar results. p-STAT3, phosphorylated STAT3; t-STAT3, total STAT3. p-Akt and p-Erk means phosphorylated Akt and Erk, respectively. C, semiquantitative analyses of expression of pSTAT3 in (B) by using densitometer. D, let-7c expression in esophageal cancer tissue is significantly lower than that of noncancerous tissue. E, IL-6 mRNA expression in cancer tissue is significantly higher than that of noncancerous tissue, determined by real-time RT-PCR. F, IL-6 mRNA expression correlated inversely with let-7c expression in noncancerous tissue ($n = 20$) and esophageal cancerous tissue ($n = 40$). G, schematic overview of relationship between let-7 and IL-6/STAT3 pathway in chemoresistance. IL-6 expression is upregulated after cisplatin exposure in esophageal cancer cells. In autocrine manner (although paracrine manner may also exist), increased expression of IL-6 upregulates phosphorylation of p-STAT3, resulting in antiapoptosis and chemoresistance. Let-7 restores sensitivity to cisplatin through repressing IL-6/p-STAT prosurvival pathway by inhibiting directly IL-6 expression.

results also showed that the effect of let-7 expression on chemosensitivity of esophageal cancer is mediated through let-7–induced repression of the IL-6/STAT3 pathway, which is prosurvival pathway activated through exposure to genotoxic agents such as cisplatin.

A few studies have reported the clinical use of miRNA expression for prediction of response to chemotherapy. Yang and colleagues (20) conducted miRNA microarray in 69 patients with epithelial ovarian cancer who had received cisplatin-based chemotherapy and reported significantly reduced let-7i expression in chemotherapy-resistant patients. They confirmed the clinical relevance of let-7i as a biomarker to predict chemotherapy response in a validation set of another 72 patients. However, the underlying mechanism of the involvement of let-7i expression in chemosensitivity of ovarian cancer was not clarified in their study. Another study by Nakajima and colleagues (21), which evaluated the expression of several miRNAs in 46 patients with recurrent or residual colon cancer, showed that upregulation of miR-181b and let-7g was significantly associated with poor response to 5-FU–based antimetabolite S-1. However, their finding of the correlation between high expression of let-7 and poor response to chemotherapy is different from our results.

The involvement of let-7 family in chemosensitivity has been examined in several *in vitro* studies. In pancreatic cancer cells, the expression of let-7b,c,d,e was significantly reduced in gemcitabine-resistant cancer cells, and upregulation of let-7 expression resulted in the reversal of epithelial–mesenchymal transition in gemcitabine-resistant cancer cells (22). In hepatocellular carcinoma cells, let-7 inhibited Bcl-xL expression, which is an antiapoptotic member of the Bcl-2 family and known to induce apoptosis in cooperation with anticancer drugs that target Mcl-1, antiapoptotic Bcl-2 protein (23). In oral cancer cells, let-7d negatively regulated EMT expression by targeting Twist and Snail and played an

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important role in modulating the sensitivity to chemotherapy such as cisplatin and 5-FU (24). In the present study, let-7 expression modulated the chemosensitivity to genotoxic chemotherapy in esophageal cancer through the IL-6/STAT3 pathway.

IL-6 is an inflammatory cytokine known to be released from macrophages and T lymphocytes as well as from cancer cells (25). Previous studies indicated that IL-6 is associated with resistance to chemotherapy in a variety of malignancies. In ovarian cancer, Wang and colleagues (26) reported that autocrine production of IL-6 confers resistance to cisplatin and paclitaxel. Iliopoulos and colleagues (18) reported that IL-6 plays a pivotal role in chemoresistance by inducing the conversion of non–cancer stem cells to cancer stem cells in breast cancer cells. With regard to esophageal cancer, one recent study showed that intracellular IL-6 expression after cisplatin exposure is associated with reduced sensitivity to cisplatin treatment and that knockdown of IL-6 expression restored sensitivity to cisplatin treatment. In the present study, we showed that esophageal cancer cells release IL-6 after exposure to cisplatin and that IL-6 activated prosurvival JAK/STAT3 pathway in an autocrine manner, leading to cisplatin resistance. On the other hand, another recent report by Gilbert and Hemann (27) showed that IL-6 secreted from endothelial cells after treatment with doxorubicin created chemoresistant niche and is involved in increased resistance to DNA damaging agents in paracrine manner. Indeed, we showed in this study that let-7 repressed IL-6 activation in esophageal cancer cells in an autocrine manner during chemotherapy, but we think that let-7 can inhibit IL-6 production from the surrounding normal cells such as fibroblasts, endothelial cells, and macrophages. Further studies are needed to clarify whether let-7 represses paracrine IL-6 signal in the surrounding normal tissues in addition to its effect on autocrine IL-6 production from cancer cells.

In this study, transfection of let-7c resulted in a significant reduction in phosphorylated STAT3 in the cells, but it did not induce any significant change in the expression of Akt and Erk. Indeed, Akt and Erk are considered to be downstream of IL-6, similar to STAT3, and to be involved in antiapoptotic pathway (26), although their expression can be regulated by upstream signals other than IL-6. For example, Akt expression is reported to be regulated by phosphoinositide 3-kinase (PI3K), mTOR, and phosphate and tensin homolog (PTEN) deleted from chromosome 10 (28–31). Erk expression is also reported to be regulated by several receptors protein tyrosine kinases and the mitogen-activated protein kinase (MAPK) pathway (32–35). One possible explanation for the lack of significant effect of let-7c transfection on Akt and Erk could be that Akt and Erk pathways are regulated mainly by signals other than IL-6 whereas STAT3 is regulated by IL-6 expression in esophageal cancer cells.

There is increasing evidence that let-7 inhibits IL-6 signaling pathway directly by targeting IL-6. Iliopoulos and colleagues (18) showed that NF-κB, Lin28, let-7, and IL-6 form an inflammatory positive feedback loop. NF-κB induces Lin28 expression, leading to inhibition of let-7 and expression of the encoding IL-6. IL-6 can itself activate NF-κB, resulting in a positive feedback loop. Another recent report showed that downregulation of let-7 promotes the expression of IL-6 and IL-10 during *Salmonella* infection. Thus, the association between let-7 and IL-6 under an inflammatory environment has been described, but this is the first time to show that the association between let-7 and IL-6 plays an important role in the sensitivity to chemotherapy for cancer. This result suggests that treatment targeting this pathway is likely to enhance the response to anticancer chemotherapy.

The present study has certain limitations. First, the clinical results were based on retrospective analysis by using biopsy samples obtained from patients who underwent preoperative chemotherapy followed by surgery at only one institution. Second, the current results that let-7 modulates the chemosensitivity in esophageal cancer through the regulation of IL-6/STAT3 pathway may be adapted into cisplatin-based chemotherapy but not other chemotherapeutic regimens that do not include cisplatin, because cisplatin-resistant cell line used in this study did not show resistance to 5-FU nor Adriamycin (data not shown). However, cisplatin-based chemotherapy is the most widely used chemotherapeutic regimen for esophageal cancer, although other chemotherapeutic regimens are used occasionally, such as taxane-based chemotherapy for esophageal cancer which has low expression of let-7. Third, before one can apply the findings that let-7 expression can be used clinically to predict the response of esophageal cancer to chemotherapy, we need to validate this result in a prospective multicenter clinical trial.

In summary, we showed that evaluation of let-7 b and let-7c expression before treatment is potentially useful to predict the response to chemotherapy in patients with esophageal cancer. Moreover, the results also showed that the effect of let-7 expression on chemosensitivity is mediated though downregulation of IL-6/STAT3 pathway. Further studies are needed to explore the therapeutic potential of the let-7/IL-6/STAT3 pathway in genotoxic anticancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Sugimura, H. Miyata, R. Hamano, M. Mori, Y. Doki

Development of methodology: K. Sugimura, H. Miyata, R. Hamano, M. Mori, Y. Doki

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Sugimura, H. Miyata, K. Tanaka, R. Hamano, T. Takahashi, Y. Kurokawa, M. Yamasaki, K. Nakajima, S. Takiguchi, M. Mori, Y, Doki

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Sugimura, H. Miyata, K. Tanaka, M. Yamasaki, M. Mori

Writing, review, and/or revision of the manuscript: K. Sugimura, H. Miyata, K. Tanaka, R. Hamano, T. Takahashi, Y. Kurokawa, M. Yamasaki, K. Nakajima, S. Takiguchi, M. Mori, Y, Doki

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Sugimura, H. Miyata, T. Takahashi, M. Yamasaki

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Study supervision: K. Sugimura, H. Miyata, K. Tanaka, R. Hamano, T. Takahashi, Y. Kurokawa, M. Yamasaki, K. Nakajima, S. Takiguchi, M. Mori, Y, Doki

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High expression of Lin28 is associated with tumour aggressiveness and poor prognosis of patients in oesophagus cancer

R Hamano[1], H Miyata*[,1], M Yamasaki[1], K Sugimura[1], K Tanaka[1], Y Kurokawa[1], K Nakajima[1], S Takiguchi[1], Y Fujiwara[1], M Mori[1] and Y Doki[1]

[1]*Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Suita, Yamadaoka 2-2, Osaka 565-0871, Japan*

BACKGROUND: Lin28 is a negative regulator of the tumour suppressor microRNA, let-7, suggesting its role in tumourigenesis. However, the clinical significance of Lin28 expression in oesophageal cancer has not been elucidated.
METHODS: Lin28 and Lin28B expression was examined by immunohistochemistry in 161 tissues from patients with oesophageal cancer who had undergone curative surgery. The relationship between the expressions of Lin28 and Lin28B and various clinicopathological factors was examined. *In vitro* assays were conducted to determine the role of Lin28 in aggressiveness of oesophageal cancers using oesophageal cancer cell line.
RESULTS: Lin28 and Lin28B were overexpressed in oesophageal cancer cells compared with non-cancerous epithelial cells, especially in the invasive front. High expression of Lin28 and Lin28B correlated significantly with lymph node metastasis and poor prognosis. High expression of Lin28B expression correlated significantly with low expression of let-7. Multivariate analysis also identified Lin28B expression as an independent prognostic factor. *In vitro* assays showed that the proliferative and invasive activities were significantly reduced in Lin28B-knockdown cells, compared with control cells.
CONCLUSION: High expression of Lin28 is associated with poor prognosis and high tumour aggressiveness in oesophageal cancer and these effects are mediated through increased proliferation and invasiveness of oesophageal cancer cells.
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Molecular Diagnostics

MicroRNAs (miRNAs) are a class of small nonprotein-coding RNAs that act by endogenous interference. They bind to the 3′ untranslated region of the target mRNAs, leading to translational repression or reduced stability of the mRNA (Bartel, 2004). MicroRNAs are known to have important roles in various biological processes, such as cell differentiation, cell proliferation, apoptosis, and metabolism. MicroRNAs also have emerged as central regulators of cancer (Calin and Croce, 2006). Their aberrant expression in many tumours indicates that they could function as tumour suppressors or oncogenes. Moreover, there is increasing evidence that miRNA expression is potentially useful biomarker for the diagnosis, prognosis and tailoring of therapy for patients with various cancers, but the mechanism involved in the regulation of miRNA expression is still uncertain. Recent studies have indicated that miRNA biogenesis can be regulated posttranscriptionally by *trans*-acting factors (Lee *et al*, 2003; Denli *et al*, 2004). It is reported that the biogenesis of let-7 family members, which seem to act as tumour suppressor miRNAs, is negatively regulated posttranscriptionally by Lin28 in embryonic stem cells and certain cancer cell lines (Heo *et al*, 2008; Newman *et al*, 2008; Piskounova *et al*, 2008; Viswanathan *et al*, 2008; Hagan *et al*, 2009).

*Correspondence: Dr H Miyata;
E-mail: hmiyata@gesurg.med.osaka-u.ac.jp
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Lin28 is a RNA-binding protein originally identified as a key regulator of developmental timing in *Caenorhabditis elegans* (Moss *et al*, 1997). Lin28 is a conserved cytoplasmic protein but is exported to the nucleus where it seems to regulate the translation or stability of mRNAs by localising them to P-bodies (Balzer and Moss, 2007). In mammals, Lin28 is widely expressed in embryonic stem cells and in early embryogenesis, but its expression is downregulated with differentiation (Moss and Tang, 2003). Recently, Lin28 was also used with three other factors (OCT4, SOX2, NANOG) to reprogramme human somatic fibroblasts to pluripotency (Yu *et al*, 2007). This finding suggests that Lin28 is related to stem cell function.

On the other hand, it was recently reported that Lin28 is upregulated in human tumours and functions as an oncogene promoting transformation and tumour progression (Viswanathan *et al*, 2009). Depletion of Lin28 and expression of let-7 suppressed bone metastasis, while Lin28 expression resulted in bone metastasis in mice implanted with breast tumour cells (Dangi-Garimella *et al*, 2009). Lin28B protein, a homologue of Lin28, is also overexpressed in hepatocellular carcinoma and induction of expression with exogenous Lin28B promoted cancer cell proliferation (Guo *et al*, 2006). Lin28B is also induced by Myc and plays an important role of Myc-dependent cellular proliferation (Chang *et al*, 2009). Thus, altered expression of Lin28 seems to promote tumourigenesis and can be associated with advanced malignancy.

In this study, we analysed the expression of Lin28 and Lin28B, a homologue of Lin28, in oesophageal cancer by immunohistochemistry and determined the relationship between their expression and various clinicopathological parameters including prognosis of patients. Moreover, in *in vitro* studies, we also examined the relationship between Lin28B expression and aggressiveness of oesophageal cancer cells using oesophageal cell lines.

PATIENTS AND METHODS

Patients and tissue samples

All 161 tissue samples were obtained from patients who underwent radical oesophagectomy with lymph node dissection for thoracic oesophageal cancers between 2000 and 2006 at the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University. Informed consent was obtained from each patient at that of surgery. Of these patients, 94 received preoperative chemotherapy followed by surgery while the remaining 67 patients underwent surgery without preoperative therapy. The preoperative chemotherapeutic regimen was cisplatin at $70\,mg\,m^{-2}$, adriamycin at $35\,mg\,m^{-2}$ (by rapid intravenous infusion on day 1), and 5-FU at $700\,mg\,m^{-2}$ (by continuous intravenous infusion on day 1 through day 7) (Miyata *et al*, 2009). Two courses of chemotherapy were used, separated by a 4-week interval. The median duration of follow-up was 32.7 months (range, 3.1–97.9 months), and 72 patients (44.7%) died during the follow-up period.

Immunohistochemistry

A staining score of Lin28 and Lin28B was calculated based on the proportion of immunostained cancer cells to that of all cancer cells in three fields of view. The expression level was categorised as high (staining score >50%) or low (staining score ⩽50%).

For immunohistochemistry, $4\,\mu m$-thick sections cut from formalin-fixed, paraffin-embedded (FFPE) tissue blocks were deparaffinised and rehydrated using xylene and serial dilutions of ethanol. Antigen retrieval was performed in $1\,mmol\,l^{-1}$ sodium citrate buffer (pH 6.0 for Lin28, pH9.0 for Lin28B) by autoclave treatment at 121°C for 15 min, and then the sections were incubated with goat serum for 20 min to block nonspecific binding followed by incubation with the primary polyclonal rabbit antibody; anti-Lin28 (1:200 dilution; Proteintech, Chicago, IL, USA) at room temperature for 2 h and anti-Lin28B (1:50 dilution; Cell Signaling, Danvers, MA, USA) overnight at 4°C. After incubation with anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 20 min, the antigen–antibody complexes were visualised using VECTASTAIN ABC kit (Vector Laboratories) according to the protocol supplied by the manufacturer. The sections were counterstained with haematoxylin. All sections were examined independently by two coauthors (R Hamano and H Miyata), who were blinded to the clinical information.

Clinical and histopathological evaluation of response to chemotherapy

The clinical response to chemotherapy was categorised according to the criteria of the World Health Organization response criteria for measurable disease (Miller *et al*, 1981) and the Japanese Society for Esophageal Diseases (Japan Esophageal Society, 2009b). A complete response was defined as total regression of the primary tumour and its disappearance on CT scan and/or PET scan and endoscopy. A partial response (PR) was defined as >50% reduction in primary tumour size and lymph node metastasis, as confirmed by CT scan. Progressive disease (PD) was defined as >25% increase in the size of the primary tumour or the appearance of new lesions. Cases that did not meet the criteria of PR or PD were defined as stable disease.

For histopathological assessment, serial $4\,\mu m$-thick tissue sections of the primary tumour and lymph nodes were cut from the surgical specimens, fixed with 10% buffered formalin, embedded in paraffin, and stained with haematoxylin and eosin. The extent of viable residual carcinoma at the primary site was assessed semiquantitatively, based on the estimated percentage of viable residual carcinoma in relation to the macroscopically identifiable tumour bed that was evaluated histopathologically. Briefly, the percentage of viable residual cancer cells within the total cancerous tissue was assessed as follows: grade 3, no viable residual tumour cells; grade 2, <2/3 residual tumour cells; grade 1b, 1/3–2/3 residual tumour cells; grade 1a, >2/3 residual tumour cells; grade 0, no significant response to chemotherapy (Miyata *et al*, 2009; Japan Esophageal Society, 2009a).

Cell lines and culture conditions

Five established cell lines derived from oesophageal squamous cell carcinoma (TE-1, -8, -10, -13, -15) were obtained from the Riken Cell Bank (Tsukuba, Japan). All cell lines were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Sigma-Aldrich Co., St Louis, MO, USA) and 1% penicillin/streptomycin (Life Technologies Inc.), under a humidified atmosphere with 5% CO_2 at 37°C.

Immunoblotting

Adherent cells grown to 50–80% confluence were washed with ice-cold phosphate-buffered saline and lysed in RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) or Sample buffer (Wako Pure Chemical Industries, Osaka, Japan) and a cocktail of phosphatase inhibitors (Thermo Fisher Scientific Inc.) on ice. Lysates were spun and the supernatant was collected. Equal amounts of cell extracts ($15\,\mu g$) were fractionated by SDS–PAGE gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto hydrophobic polyvinylidene difluoride membranes (ImmobilonP, Millipore, Bedford, MA, USA). The membranes were blocked by incubation in 5% milk followed by incubation overnight at 4°C with the primary antibodies, and then with the secondary antibodies for 1 h at room temperature. The following antibodies were used in this study; anti-actin (dilution, 1:1000, Sigma-Aldrich Co.), and anti-Lin28B (dilution, 1:1000, Abcam, Cambridge, MA, USA). Immune complexes were detected using Detection Kit (GE Healthcare, Buckinghamshire, UK).

Small interfering RNA transfection

Cells were cultured to 60–80% confluence and transfected with $1\,\mu M$ of small interfering RNAs (siRNAs) that targeted human Lin28B (Si-Lin28B) or negative control oligonucleotides (Applied Biosystems, Foster City, CA, USA) using siPORT NeoFX Transfection Agent (Ambion, Austin, TX, USA) according to the protocol provided by the manufacturer. After transfection, the cells were cultured for 72 h and intermediate samples were collected at 24 and 48 h and analysed by immunoblotting and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

MTT cell proliferation assay

The MTT assay was used to assess proliferative activity. After siRNA transfection, cells were seeded into 96-well plates at 5×10^3 per well and incubated overnight under standard culture condition. Following incubation for 24, 48, 72, and 96 h, $10\,\mu l$ of MTT solution was added to each well and the plates were incubated for another 3 h at 37°C, and formazan crystals were dissolved with $100\,\mu l$ of 0.04 N HCl-isopropanol. The absorbance of individual wells was read at 550 nm test wavelength and 655 nm reference wavelengths using a microplate reader (Bio-Rad Laboratories).

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Cell proliferation activities of siRNA transfected cells and negative control transfected cells were determined by absorbance values.

Invasion assay

In vitro cell invasion was assayed using the BioCoat Matrigel Invasion Chambers (Becton Dickinson Biosciences, Sparks, MD, USA) using the procedure recommended by the manufacturer. Briefly, the transfected cells were harvested and placed in the upper chamber (2.5×10^5 cells per well) in serum-free medium. After incubation at 37°C for 48 h to allow invasion of the Matrigel-coated chamber, the invaded cells on the lower surface were fixed and stained using Diff-Quik stain kit (Dade Behring Inc., Newark, DE, USA), whereas the noninvading cells on the upper surface were scraped and washed away. Finally, the number of invaded cells was counted under a microscope in nine random fields ($\times 200$).

RNA isolation from FFPE specimens

Total RNA was isolated from the FFPE tissue specimens using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion) according to the instructions supplied by the manufacturer. Briefly, each FFPE tissue block was cut into 20-μm thick pieces, and four slices were placed in a centrifuge tube. To liquefy the paraffin, 100% xylene and 100% ethanol was added to each tube. After centrifugation, the precipitated samples were air dried and treated with protease in heat blocks for 3 h at 50°C. Then, each sample was treated with isolation reagent and filtered. Each filter was treated with DNase and incubated for 30 min at room temperature. After washing the filter with washing reagents, it was treated with warmed Elution Solution and centrifuged to pass the mixture through the filter. The eluate contained the isolated RNA.

Quantitative real-time reverse transcription-PCR

The complementary DNA was synthesised from 10 ng of total RNA using the TaqMan miRNA Reverse Transcription Kit and specific stem-loop reverse transcription primers (Applied Biosystems) according to the protocol provided by the manufacturer. The reverse transcription was set at these conditions: 16°C for 30 min followed by 40°C for 30 min and 85°C for 5 min. Real-time PCR reaction was performed using TaqMan Universal PCR master mix No AmpErase UNG and TaqMan miRNA specific PCR primers (Applied Biosystems). A 20 μl of the reaction product was incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C at 1 min using ABI PRISM 7900HT (Applied Biosystems). The miRNA expression value was expressed relative to that of RNU48 and analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Hamano *et al*, 2011).

Statistical analysis

All data are expressed as mean ± s.d. The relationship between miRNA expression and each clinicopathological variable was analysed by χ^2 test, Fisher's exact test, or Mann-Whitney *U*-test. Time to recurrence was defined as the time interval between the date of surgery and the date of diagnosis of first recurrence or last

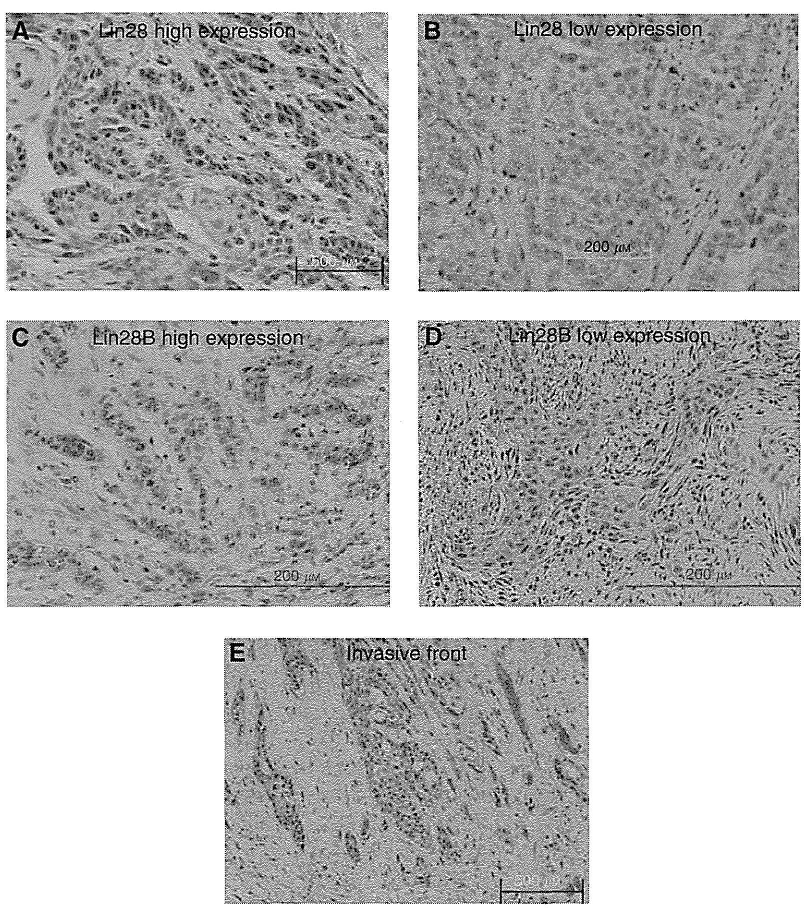

Figure 1 Lin28 and Lin28B immunostaining in oesophageal cancer. (**A**) Lin28 positively stained cells. (**B**) Lin28 negatively stained cells. (**C**) Lin28B positively stained cells. (**D**) Lin28B negatively stained cells. (**E**) The carcinoma cells at the invasive front.

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date of follow-up if recurrence was not observed. Overall survival time was censored at the date of the last follow-up if death did not occur. For survival analysis, the Kaplan–Meier method was used to assess survival time distribution according to miRNA expression level and the log-rank test was used to examine the differences between groups. A P-value of <0.05 denoted the presence of statistically significant difference between groups. All statistical analyses were performed with JMP ver. 8.0 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

High expression of Lin28 and Lin28B in oesophageal cancer cells

First, we determined the expression pattern of Lin28 and Lin28B in cancerous and non-cancerous tissues from patients with oesophageal cancer. Lin28 staining was predominantly detected in the nuclei of cancer cells, though cytoplasmic staining was also evident

Table 1 Correlation between Lin28 expression and various clinicopathological features of patients with oesophageal cancer

	Lin28 expression		
	High	**Low**	***P*-value**
n	72	89	
Sex			
Male/female	63/9	81/8	0.607
Age (years)			
Mean ± s.d.	61.99 ± 9.5	64.23 ± 7.7	0.101
pStage			
0	0	2	0.068
1	8	16	
2	21	26	
3	19	26	
4	24	19	
Histology			
Well SCC	18	14	0.413
Moderate SCC	32	49	
Poor SCC	15	16	
Other	7	10	
pT			
0	0	2	0.249
1	14	24	
2	13	11	
3	35	45	
4	10	7	
pN			
0	19	38	0.046
1	53	51	
Mean ± s.d.	6.68 ± 16.9	2.73 ± 4.3	0.035
pM			
0	47	70	0.042
1	3	0	
1a	5	9	
1b	17	10	
Lymphatic invasion			
+	63	66	0.047
−	9	23	
Venous invasion			
+	34	42	0.999
−	38	47	
Preoperative chemotherapy			
+	46	48	0.260
−	26	41	
Curative surgery			
Yes	64	85	0.137
No	8	4	

Abbreviation: SCC = squamous cell carcinoma.

Table 2 Correlation between Lin28B expression and various clinicopathological features of patients with oesophageal cancer

	Lin28B expression		
	High	**Low**	***P*-value**
n	66	71	
Sex			
Male/female	57/9	65/6	0.416
Age (years)			
Mean ± s.d.	62.3 ± 9.5	62.8 ± 7.7	0.730
pStage			
0	0	2	0.003
1	3	13	
2	17	21	
3	23	21	
4	23	14	
Histology			
Well SCC	15	12	0.358
Moderate SCC	29	42	
Poor SCC	15	11	
Other	7	6	
pT			
0	0	2	0.005
1	6	21	
2	14	7	
3	34	38	
4	12	3	
pN			
0	14	29	0.017
1	52	42	
Mean ± s.d.	5.17 ± 9.2	4.54 ± 15.3	0.773
pM			
0	44	55	0.135
1	1	0	
1a	7	6	
1b	14	10	
Lymphatic invasion			
+	61	50	0.002
−	5	20	
Venous invasion			
+	36	28	0.088
−	30	43	
Preoperative chemotherapy			
+	45	39	0.119
−	21	32	
Curative surgery			
Yes	56	70	0.004
No	10	1	

Abbreviation: SCC = squamous cell carcinoma.

in some cancer cells (Figure 1A and B). Lin28B staining was also predominantly detected in the nuclei of cancer cells (Figure 1C and D). This finding is in agreement with the results of a previous study of Lin28 expression in the cytoplasm and its transportation to the nucleus (Balzer and Moss, 2007). The carcinoma cells at the invasive front tended show strong staining for Lin28 and Lin28B compared with those in other areas (Figure 1E). On the other hand, in non-cancerous tissue, differentiated cells were not stained although the cytoplasm of some basal cells was stained weakly for Lin28 and Lin28B.

High expression of Lin28 is associated with poor prognosis

Tables 1 and 2 summarise the relationship between Lin28 and Lin28B expression and various clinicopathological parameters. There were no significant relationships between Lin28 expression and tumour differentiation or tumour depth. However, high expression of Lin28 correlated significantly with lymph node metastasis ($P = 0.035$) and lymphatic vessel invasion ($P = 0.047$). Similar to Lin28, high expression of Lin28B correlated significantly with lymph node metastasis ($P = 0.017$) and lymphatic vessel invasion ($P = 0.002$). Moreover, the expression of Lin28B significantly correlated with tumour depth ($P = 0.005$).

High expression of Lin28 correlated significantly with shortened survival including both overall survival and disease-free survival (Figure 2A and B). High expression of Lin28B also correlated significantly with shortened survival (Figure 2C and D). Furthermore, multivariate analysis identified Lin28B expression as an independent prognostic factor, along with the number of metastatic lymph nodes (Table 3). These results suggest that Lin28 and Lin28B may influence the malignant potential of oesophageal cancer. However, Lin28 expression did not correlate with clinical and pathological responses to preoperative chemotherapy (Table 4).

Inhibition of Lin28B expression regulates cellular behaviour

The above results suggest that Lin28 and Lin28B are potentially associated with aggressiveness of oesophageal cancer. In the next series of studies, *in vitro* experiments were conducted to examine the effect of these expressions on the malignant potential of oesophageal cancer cells. First, we screened several oesophageal cancer cell lines, and found that some cell lines express Lin28B, while expression of Lin28 is quite low in all cell lines examined (data not shown). Thus, to study the effects of expression of Lin28B on cellular proliferation, its expression was knocked down by transfecting si-Lin28B in TE-13 oesophageal cancer cells (Figure 3A). The proliferative activity of Lin28B-knockdown cells was significantly reduced compared with that of control cells (Figure 3B). Second, the invasion assay was conducted to assess the role of Lin28B in lymph node metastasis by invasion to lymphatics. The invasive activity of Lin28B-knockdown cells was clearly reduced compared with that of negative control cells (Figure 3D and E). In another oesophageal cancer cell line, TE-10, the reduced proliferation and invasive activity of Lin28B-knockdown cells were confirmed (Figure 3C and F).

Relationship between Lin28 expression and let-7 expression

Lin28 is described as a negative regulator of let-7 biogenesis (Heo *et al*, 2008; Viswanathan *et al*, 2008). We investigated the relationship between expression of Lin28 and Lin28B and let-7 expression. We found significant relationship between let-7 expression and Lin28B expression, but not Lin28 expression in the surgical specimens of oesophageal cancer (Figure 4A–D). *In vitro* assay showed that let-7 was upregulated in cultured Lin28B-knockdown oesophageal cancer cells, compared with control cells (Figure 4E). This finding is consistent with the results

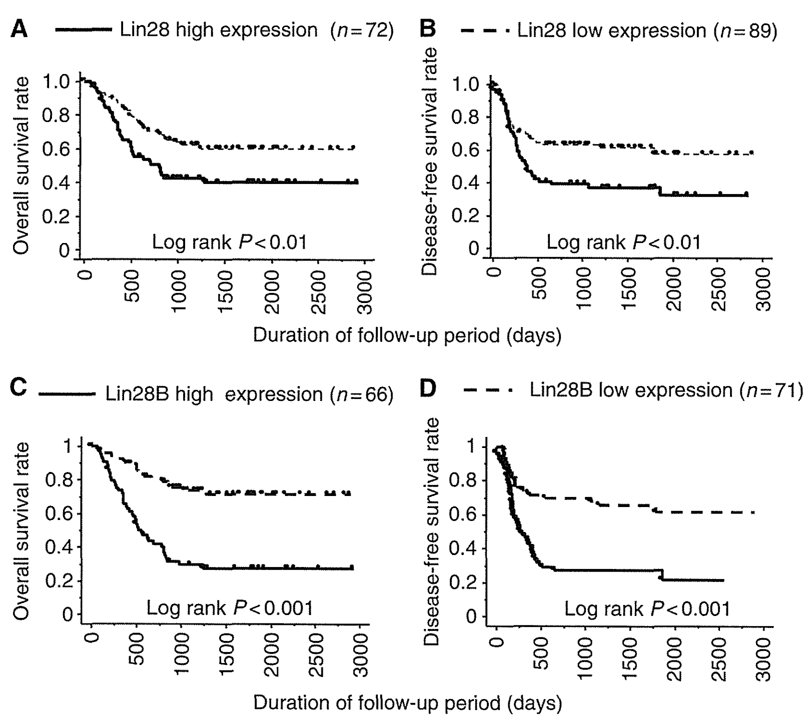

Figure 2 Correlation between Lin28/Lin28B expression and survival of 161 patients with oesophageal cancer. High expression of Lin 28 (**A** and **B**)/ Lin28B (**C** and **D**) correlated with shortened survivals.

Table 3 Results of univariate and multivariate Cox's models for disease-free survival

	n	%	Univariate analysis		Multivariate analysis	
			HR (95% CI)	P-value	HR (95% CI)	P-value
Sex						
Males	144	89.4	0.986 (0.380–2.563)	0.9774		
Females	17	10.6				
Age						
>65 years	76	47.2	1.616 (0.469–1.560)	0.6104		
⩽65 years	85	52.8				
pT						
3–4	97	60.2	10.354 (0.193–0.680)	0.0013	1.242 (0.350–1.854)	0.6105
0–2	64	39.8				
pN						
1	104	64.6	30.630 (0.073–0.311)	<0.001	4.310 (0.094–0.570)	0.001
0	57	35.4				
Differentiation						
Other	129	80.1	1.976 (0.271–1.249)	0.1599		
Well	32	19.9				
Curability						
R1–2	12	7.5	3.994 (1.013–1.072)	0.0457	1.319 (0.140–4.109)	0.7484
R0	149	92.5				
Lin28B expression						
Low	71	51.8	0.166 (0.079–0.349)	<0.001	0.193 (0.087–0.432)	<0.001
High	66	48.2				

Abbreviations: HR = hazard ratio; 95% CI = 95% confidence interval.

of the previous study showing a relationship between Lin28B and let-7 (King *et al*, 2011).

DISCUSSION

Lin28 is a negative regulator of let-7 family, which may act as a tumour suppressor miRNA, suggesting that Lin28 could contribute to tumourigenesis. The present study demonstrated that high expression of Lin28 and Lin28B is associated with lymph node metastasis and poor prognosis of patients with oesophageal cancers. *In vitro* studies confirmed that Lin28B expression was associated with aggressiveness of oesophageal cancer through increased proliferation and invasive activities in oesophageal cancer cells.

Recent studies suggest that Lin28 functions as an oncogene promoting malignant transformation and tumour progression (Viswanathan *et al*, 2009). Indeed, several recent reports demonstrated that Lin28 expression correlates with survival of patients with malignant diseases (Guo *et al*, 2006). In ovarian cancer, patients with high Lin28B expression had shorter progression-free and overall survival times than those with low Lin28B expression (Lu *et al*, 2009). In another recent report, high Lin28B staining intensity in stage I/II colon cancers correlated with reduced survival and increased probability of tumour recurrence (King *et al*, 2011). Our result of the correlation between high expression of Lin28 and Lin28B and poor prognosis of patients with oesophageal cancers is compatible with the above studies. Thus, Lin28 expression may be clinically relevant prognostic marker in various malignancies including oesophageal cancer.

The present study demonstrated that Lin28 expression is associated with tumour aggressiveness through increased proliferation of oesophageal cancer cells. One recent study demonstrated that Lin28B is necessary and sufficient for Myc-mediated let-7 repression, and that Lin28B has an important role in Myc-dependent cellular proliferation (Chang *et al*, 2009). Another study showed that high expression of Lin28 and Lin28B correlated with low let-7 expression and upregulation of let-7 target such as HMGA2 and Myc, and that knockdown of Lin28B expression impaired cellular proliferation (Viswanathan *et al*, 2009). Thus, Lin28 may increase proliferation of oesophageal cancer cells by directly inhibiting let-7 expression and subsequently upregulating HMGA2 and Myc, which are targets of let-7.

In this study, high expression of Lin28 correlated significantly with lymph node metastasis in patients with oesophageal cancers.

Table 4 Relationship between Lin28 expression and response to preoperative chemotherapy in patients with oesophageal cancer

	Lin28 expression			Lin28B expression		
	High n = 46	Low n = 48	P-value	High n = 45	Low n = 39	P-value
Clinical response						
CR	0	3	0.621	1	2	0.126
PR	24	22		19	21	
SD–PD	22	23		25	16	
Pathological response						
2	3	6	0.210	1	4	0.103
1b	8	11		7	10	
1a	20	19		22	15	
0	15	12		15	10	

Abbreviations: SD = stable disease; PD = progressive disease; PR = partial response; CR = complete response.

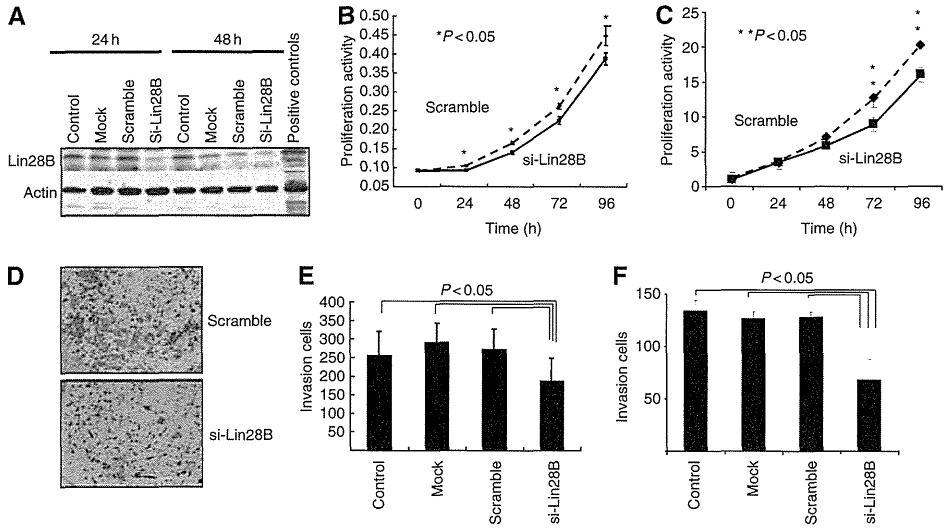

Figure 3 Proliferative and invasive activities of Lin28B-knockdown cells. (**A**) Western blotting to confirm reduced Lin28B expression following transfection of si-Lin28B in TE-13. (**B**) Proliferative activities of Lin28B-knockdown cells and control cells in TE-13. (**C**) Proliferative activities of Lin28B-knockdown cells and control cells in TE-10. (**D**) Invasive activities of Lin28B-knockdown cells and control cells in TE-13. (**E**) Quantitative analysis of invasive activity in TE-13 (data are mean ± s.d. of three experiments). (**F**) Quantitative analysis of invasive activity in TE-10 (data are mean ± s.d. of three experiments).

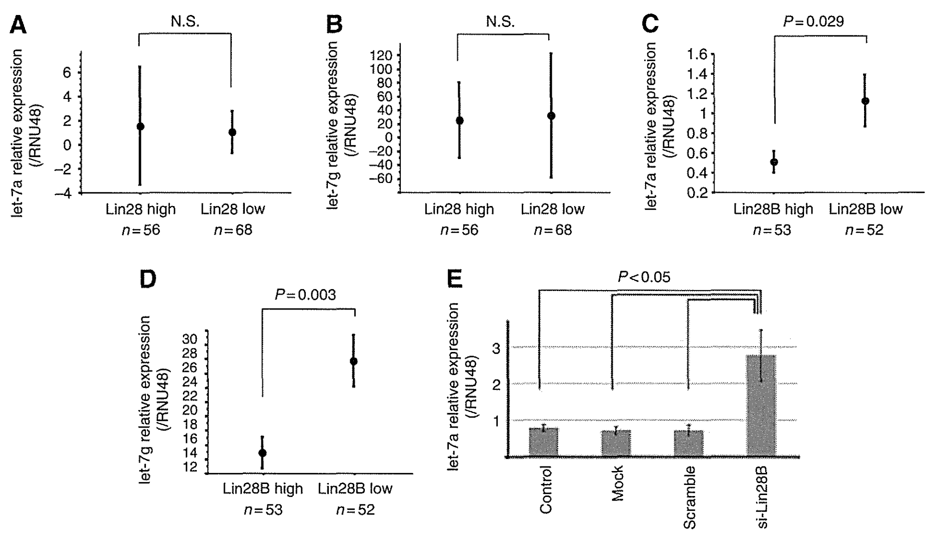

Figure 4 Relationship between Lin28B expression and let-7 expression in oesophageal cancer. (**A** and **B**) Analysis of surgical specimens showed no significant relationship between Lin28 expression and let-7 expression, determined by real-time RT–PCR. (**C** and **D**) Analysis of surgical specimens showed significantly relationship between Lin28B expression and let-7 expression, determined by real-time RT–PCR. (**E**) *In vitro* assay using oesophageal cancer cell showed upregulation of let-7 expression in Lin28B-knockdown cells, compared with control cells.

A recent study showed that Raf kinase inhibitory protein repressed breast tumour cell intravasation and bone metastasis in a mouse model, through inhibition of mitogen-activated protein kinase, leading to decreased transcription of Lin28 and enhanced expression of let-7 by Myc (Dangi-Garimella *et al*, 2009). These data provided the first evidence for the roles of Lin28 and let-7 expression in tumour metastasis, in addition to the regulation of tumour growth. Another recent study demonstrated that constitutive expression of Lin28B expression in colon cancer cells confers metastatic ability by showing that mice xenografted with Lin28B expressing colon cancer cells developed much more metastasis in the liver, lung, and mesenterium compared with mice of the empty vector control group (King *et al*, 2011). Thus, Lin28 expression may have an important role in cancer metastasis. In the present study, Lin28B expression was significantly associated with the invasive activity of oesophageal cancer cells. The result that high expression of Lin28 correlated with lymph node metastasis in oesophageal cancer may depend on the increased invasiveness through Lin28 expression.

Several recent studies have identified the important roles of Nanog, Sox2 and Oct3/4, which are involved in reprogramming and maintenance of stem cell function, in tumour aggressiveness

(Ben-Porath et al, 2008). Immunohistochemical analysis of gastric cancer (Lin et al, 2011) and colorectal cancer (Meng et al, 2010) showed that overexpression of Nanog correlated strongly with lymph node metastasis and poor prognosis of patients. In hepatocellular carcinoma, Sox2 and Oct4 were identified as independent prognostic factors with poorest prognosis in patients with tumours that co-expressed Sox2/Oct4 proteins (Huang et al, 2011). In oesophageal cancer, the expression of Oct3/4 and Sox2 proteins was reported to correlate with advanced cancer, which in turn correlated with poor clinical outcome (Wang et al, 2009). In our study, Lin28, a reprogramming factor, was associated with tumour aggressiveness and poor prognosis of patients with oesophageal cancer. Thus, reprogramming factors, which regulates stem cell like properties such as pluripotency and self-renewal in normal cells, may confer the high malignant potential of cancer cells.

In the present study, Lin28B expression correlated inversely with let-7 expression in oesophageal cancer cell line and human oesophageal cancers, although no such correlation was identified between Lin28 and let-7 expression. Several studies have indicated that Lin28/Lin28B is part of the regulatory network that also involves let-7. Lin28/Lin28B represses let-7, which itself represses Lin28/Lin28B by binding to the 3′UTR of Lin28/Lin28B transcripts, thus forming a double-negative feedback loop. A second feedback loop is that Lin28/Lin28B de-represses c-Myc by inhibiting let-7, and c-Myc transcriptionally activates Lin28/Lin28B (Chang et al, 2009; Dangi-Garimella et al, 2009). A third feedback loop involves NF-κB, Lin28B, let-7 and IL-6 (Iliopoulos et al, 2009). NF-κB induces Lin28B expression, leading to inhibition of let-7 and expression of the encoding IL-6 (a let-7 target). IL-6 can itself activate NF-κB, resulting in a positive feedback loop. Thus, Lin28 and let-7 may form a complex feedback loop in malignant transformation. Moreover, one recent study showed that Lin28 and Lin28B function through distinct mechanisms to block let-7 processing (Piskounova et al, 2011). Further studies are required to elucidate the roles of Lin28/Lin28B and let-7 network in oesophageal cancers.

In summary, we examined in the present study the clinical significance of Lin28 and Lin28B expression in oesophageal cancer and demonstrated that high expressions of Lin28 and Lin28B are associated with lymph node metastasis and poor prognosis of patients with oesophageal cancers. Moreover, *in vitro* studies confirmed that Lin28B expression was associated with aggressiveness of oesophageal cancer by increasing the proliferation and invasiveness of oesophageal cancer cells. Further studies are needed to confirm the role of feedback loops including Lin28/Lin28B and let-7 in oesophageal cancer.

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Original Article

Effects of Ghrelin Administration During Chemotherapy With Advanced Esophageal Cancer Patients

A Prospective, Randomized, Placebo-Controlled Phase 2 Study

Yuichiro Hiura, MD[1]; Shuji Takiguchi, MD, PhD[1]; Kazuyoshi Yamamoto, MD, PhD[1]; Tsuyoshi Takahashi, MD, PhD[1]; Yukinori Kurokawa, MD, PhD[1]; Makoto Yamasaki, MD, PhD[1]; Kiyokazu Nakajima, MD, PhD[1]; Hiroshi Miyata, MD, PhD[1]; Yoshiyuki Fujiwara, MD, PhD[1]; Masaki Mori, MD, PhD[1]; Kenji Kangawa, MD, PhD[2]; and Yuichiro Doki, MD, PhD[1]

BACKGROUND: Cisplatin reduces plasma ghrelin levels through the 5-hydroxytryptamine (5-HT) receptor. This may cause cisplatin-induced gastrointestinal disorders and hinders the continuation of chemotherapy. The authors of this report conducted a prospective, randomized phase 2 trial to evaluate the effects of exogenous ghrelin during cisplatin-based chemotherapy. **METHODS:** Forty-two patients with esophageal cancer who were receiving cisplatin-based neoadjuvant chemotherapy were assigned to either a ghrelin group (n = 21) or a placebo group (n = 21). They received either intravenous infusions of synthetic human ghrelin (3 μg/kg) or saline twice daily for 1 week with cisplatin administration. The primary endpoint was changes in oral calorie intake, and the secondary endpoints were chemotherapy-related adverse events; appetite visual analog scale (VAS) scores; changes in gastrointestinal hormones and nutritional status, including rapid turnover proteins, and quality of life (QoL) estimated with the European Organization for Research and Treatment of Cancer QoL core questionnaire (QLQ-C30). **RESULTS:** Two patients were excluded from the final analysis: One patient suspended ghrelin administration because of excessive diaphoresis, and another patient in the placebo group failed to monitor the self-questionnaire. Food intake and appetite VAS scores were significantly higher in the ghrelin group than in the placebo group (18.2 ± 5.2 kcal/kg/day vs 12.7 ± 3.4 kcal/kg/day [$P = .001$] and 6.2 ± 0.9 vs 4.1 ± 0.9 [$P < .0001$], respectively). Patients in the ghrelin group had fewer adverse events during chemotherapy related to anorexia and nausea than patients in the control group. Significant deterioration was noted after chemotherapy in the placebo group in QoL scores, appetite, nausea and vomiting, and global health status. **CONCLUSIONS:** Short-term administration of exogenous ghrelin at the start of cisplatin-based chemotherapy stimulated food intake and minimized adverse events. *Cancer* 2012;118:4785-94.

KEYWORDS: ghrelin, esophageal cancer, food intake, appetite, cisplatin-based chemotherapy.

INTRODUCTION

Neoadjuvant and/or adjuvant chemotherapy using multiple antitumor agents is an important component of any therapeutic regimen for advance-stage solid tumors.[1] Cisplatin plays a central role in the success of such multidrug chemotherapy regimens for various cancers[2]; however, it is also associated with an assortment of adverse effects, including nephrotoxicity, myelosuppression, and gastrointestinal disorders like nausea, vomiting, and appetite loss. These gastrointestinal symptoms generally are nonlethal and reversible; however, their high frequency and strength can strongly impair the patient's quality of life (QoL) and, in general, may preclude the completion of chemotherapy.

The acute phase of cisplatin-induced gastrointestinal disorders involve increased serotonin (5-hydroxytryptamine [5-HT]) secretion from enterochromaffin cells.[3] Consequently, a 5-HT3-receptor antagonist was developed and is widely used for patients with cancer who are receiving cisplatin-based chemotherapy.[4] Despite this advance, many patients still suffer from gastrointestinal disorders because of cisplatin, especially in the later phases of treatment.

Ghrelin is an endogenous ligand for the growth hormone (GH) secretagogue receptor and is secreted predominantly by gastric endocrine cells.[5] It induces dose-dependent, GH-releasing activity[5,6]; stimulates appetite and food intake; and triggers a positive energy balance through a central mechanism involving hypothalamic neuropeptides.[5-9] In rodents, ghrelin increases GH secretion, feeding, and body weight when administered centrally or peripherally.[7-9] We also reported

Corresponding author: Shuji Takiguchi, MD, PhD, Division of Gastroenterological Surgery, Department of Surgery, Graduate School of Medicine, Osaka University, 2-2, E2, Yamadaoka, Suita, Osaka 565-0871, Japan; Fax: (011) 81-6-6879-3259; stakiguchi@gesurg.med.osaka-u.ac.jp

[1]Division of Gastroenterological Surgery, Department of Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan; [2]Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan

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previously that intravenous administration of ghrelin enhanced oral feeding and diminished weight loss in patients who underwent total gastrectomy[10] and esophagectomy.[11]

In rodents, cisplatin markedly decreased plasma ghrelin concentrations, whereas the administration of exogenous ghrelin improved cisplatin-induced decreases in food intake.[12,13] These observations suggested that ghrelin also may be effective in minimizing the gastrointestinal disorders induced by cisplatin in humans, although there are huge differences in feeding activity between the 2 species. Accordingly, we undertook a randomized clinical trial to elucidate the effect of exogenous ghrelin on patients with esophageal cancer who were receiving cisplatin-based neoadjuvant chemotherapy.

MATERIALS AND METHODS

Patients

This prospective, randomized, placebo-controlled phase 2 study enrolled 42 patients with advanced esophageal cancer who received cisplatin-based neoadjuvant chemotherapy. The Human Ethics Review Committee of Osaka University School of Medicine approved the study protocol, and a signed consent form was obtained from each enrolled patient before study entry in accordance with the Declaration of Helsinki. This study was registered on the University Hospital Medical Information Network (R000005924). It began in February 2010, and enrollment of patients ended in January 2011. The eligibility criteria for the study were as follows: 1) histopathologically confirmed squamous cell carcinoma of the esophagus; 2) stage II or III disease according to criteria of the International Union Against Cancer (UICC), sixth edition[14]; 3) ages 20 to 80 years; 4) no esophageal obstruction by tumor and capacity for oral intake of soft solid foods; 5) adequate function of major organs; 6) no other active malignancy; 7) an Eastern Cooperative Oncology Group performance status (PS) of 0 or 1; and, 8) provision of written informed consent. The exclusion criteria for the study were as follows: 1) pregnant or potentially (willingly) pregnant women; 2) a past history of other chemotherapy or radiotherapy; and, 3) patients judged to be ineligible by the investigator.

A coordinating center (a section of the Department of Gastroenterological Surgery, Osaka University Medical School) was responsible for creating the treatment allocation code using a computer-generated randomization table with a statistician. Patients were randomized at a 1:1 ratio to receive intravenous infusion of either synthetic human ghrelin (3 μg/kg) or placebo (saline). Treatment allocation was arranged before the beginning of chemotherapy. The study was performed in a single-blind manner, ie, without knowledge of allocation to the patients.

Calculation of Sample Size

We estimated that oral intake of food calories during the study period in the placebo group would be 1600 ± 300 kcal/day. The power calculation was based on a 20% improvement by ghrelin administration in oral food intake calories, with a power of 85% and an α value of 5%, requiring at least 17 patients per study group. Assuming that approximately 20% of patients in each group would not complete the study, the initial proposal aimed to recruit 20 patients in each group.

Neoadjuvant Chemotherapy Regimen

The enrolled patients received cisplatin-based chemotherapy. This was a regimen consisting of either 5-fluorouracil, cisplatin, and doxorubicin (ACF)[3,15] or 5-fluorouracil, cisplatin, and docetaxel (DCF).[16] Both regimens entailed 2 treatments every 4 weeks. Specifically, the ACF regimen comprised cisplatin (70 mg) and doxorubicin (35 mg) on day 1 and a continuous infusion of 5-fluorouracil (700 mg/day) for 7 days, whereas the DCF regimen comprised cisplatin (70 mg) and docetaxel (70 mg) on day 1 and then a continuous infusion of 5-fluorouracil (700 mg/day) for 5 days. Supportive therapy and prophylaxis against expected side effects was provided. All patients were premedicated with intravenous ramosetron hydrochloride (0.3 mg), a representative 5-HT3 receptor antagonist. This was infused 1 hour before the administration of cisplatin on day 1 and every morning thereafter on days 2 through 7 (ACF regimen) or days 2 through 5 (DCF regimen). Hypersensitivity reactions were treated prophylactically with intravenous dexamethasone (8 mg), which was infused 1 hour before the administration of cisplatin. Adequate hydration was ensured before and after cisplatin infusion. Additional antiemetics or steroid preparations were recommended in case of grade 3 or greater anorexia, nausea, and vomiting according to toxicity grading criteria from the Common Terminology Criteria for Adverse Events version 4.0 (CTCAE).[17] After completion of the second cycle of neoadjuvant chemotherapy, the patient underwent curative resection, ie, subtotal esophagectomy with reconstruction by gastric tube, together with 2-field or 3-field lymphadenectomy.[18]

Evaluation of Adverse Events and Criteria for Dose Modifications

Adverse events were evaluated each day of chemotherapy and were scored by the most severe event in the first cycle (days 1-28) based on the toxicity grading criteria from the