Let-7 expression is a significant determinant of response to chemotherapy through the regulation of IL-6/STAT3 pathway in esophageal squamous cell carcinoma.

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Abbreviations

IL-6, interleukin-6
STAT3, signal transducer and activator of transcription 3

miRNA, microRNA

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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 microRNA expression using biopsy samples before treatment. Of the several microRNAs 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 IL-6/STAT3 pathway in esophageal cancer. This result should help doctors and scientists dealing with chemotherapy for gastrointestinal cancers including esophageal cancer.
Abstract

Purpose: Cisplatin-based chemotherapy is widely used for esophageal cancer, sometimes in combination with surgery/radiotherapy, but poor response to chemotherapy is not uncommon. The aim of this study was to examine whether microRNA (miRNA) expression is useful to predict the response to chemotherapy in patients with esophageal cancer.

Experimental Design: Using pretreatment biopsy samples from 98 patients with esophageal cancer who received preoperative chemotherapy, we measured the expression level of several miRNAs whose expression was altered in cisplatin-resistant esophageal cancer cell lines compared with those parents cell lines, and examined the relationship between the microRNA expression and response to chemotherapy. In vitro assays were performed to clarify the mechanism of miRNA-induced changes in chemosensitivity.

Results: The expression levels of 15 miRNAs were altered in cisplatin-resistant cells. Of these, low expression of let-7b and let-7c in before-treatment biopsies from 74 patients of the training set, correlated significantly with poor response to chemotherapy, both clinically and histopathologically. Low expression of let-7c also correlated with poor prognosis (p=0.032). The relationship between let-7b and let-7c expression and response to chemotherapy was confirmed in the other 24 patients of the validation set. In vitro assay, transfection of let-7c restored sensitivity to cisplatin and increased rate of apoptosis after exposure to cisplatin. Let-7c directly repressed cisplatin-activated IL-6/STAT3 prosurvival pathway.

Conclusions: Let-7 expression in esophageal cancer can be potentially used to predict the response to cisplatin-based chemotherapy. Let-7 modulates the chemosensitivity to
cisplatin through the regulation of IL-6/STAT3 pathway in esophageal cancer.
Introduction

Despite recent advances in surgical techniques and perioperative management, the prognosis of patients who undergo surgery alone for esophageal cancer remains poor(1). Neoadjuvant chemotherapy or chemoradiotherapy followed by surgery has emerged as a promising strategy for advanced esophageal cancer and in fact, good responders to such preoperative therapy show better survival(2)(3). However, the reported response rate to cisplatin-based chemotherapy, which is widely used for esophageal cancer, is only modest, ranging from 25 to 48%(4-7), and non-responders likely receive no survival benefit(8). The ability to predict the response to chemotherapy before treatment should limit the application of chemotherapy to selected patients who are likely to show some benefits, and allow tailoring such therapy to the individual patient with esophageal cancer.

MicroRNAs (miRNAs) are non-coding RNAs of approximately 22 nucleotides in size, and act by repressing the translation of target mRNA by binding to the 3’ untranslated region of those mRNAs(9). MiRNAs exist stably in various tissues and play pivotal roles in differentiation and development (10). In addition, aberrant expression of miRNAs is reported in various types of cancers. In esophageal cancer, miR-21 and miR-93 are reported to be up-regulated while miR-375, miR-27b, miR-203,miR-205 and let-7c are down-regulated(11)(12). Recent studies also showed the involvement of several miRNAs in resistance to anticancer treatment including chemotherapy and radiotherapy. Giavannetti et al. (13) reported that overexpression of miR-21 was associated with poor outcome in gemcitabine-treated patients with pancreatic cancer. In our previous study using residual tumor after chemotherapy, we demonstrated the involvement of upregulated miR-200c expression in chemoresistance in esophageal cancer.
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**

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**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, Japan. Informed consent was obtained from each patient before participation in this study. These 98 patients were divided at random into two 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 two courses of 5-fluorouracil, cisplatin, and adriamycin, using the following protocol: Cisplatin was administered at 70 mg/m\(^2\),
adriamycin was administered at 35 mg/m² intravenously on day 1 and 5-FU was administered continuously from day 1 to day 7 at 700 mg/m²/day. 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 two groups: the first 74 patients were categorized as the training set while the second group of 24 patients was categorized as the validation set (Supplementary Table 1).

**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) representing total regression of the tumor. Partial response (PR) represented more than 50\% reduction in primary tumor size on 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 while the SD and PD were grouped as non-responders. The clinical response was assessed retrospectively by two investigators (K.S. and H.M.) in a blinded fashion. The histopathological 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 3: no viable residual tumor cells, grade 2: less than two-third residual tumor cells, grade 1: more than two-third residual tumor cells, grade 0: no significant response to chemotherapy. The histopathological response was assessed retrospectively by two investigators (K. S. and K.T..) 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 (Tsukuba, Japan). All cells were cultured in RPMI media 1640 (Life Technologies, New York, NY), containing 10% fetal bovine serum (FBS) (Sigma-Aldrich Co, St, Louis, MO) and 1% penicillin / streptomycin (Life Technologies), in a humidified atmosphere under 5% CO₂ 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, Osaka, Japan), as described previously(14). The cultured cells were exposed cisplatin at an initial concentration of 2 μM. Three days later, the cells were cultured in cisplatin-free medium until confluence. Next, cisplatin concentration was increased by 2-3 folds. This cycle was repeated until cisplatin concentration reached 35 μM.

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 were 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.

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**RT-PCR and Taqman miRNA Assay**

To 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, RNU48 ID:001006. The PCRs were performed 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 performed using the $2^{-\Delta\Delta Ct}$ method(17).

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**IL-6 quantitative RT-PCR**

For reverse transcriptase reaction, the Reverse Transcription System (Promega, Tokyo, Japan) was used according to the protocol provided by the manufacturer. Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed using designed oligonucleotide primers and Light Cycler (Roche Diagnostics, Mannheim, Germany),
and the amount of 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’ IL-6 reverse primer 5’-ATCTGAGGTGCCCATGCTAC-3’ GAPDH forward primer 5’-CAACTACATGGTTTACATGTTC-3’ GAPDH reverse primer 5’-AAATGAGCCCCAGCCTTC-3’.

miRNA microarray

The miRNA expression profiling was performed with 1000 ng of RNA extracted from two esophageal cell lines (TE8 and TE10) and the corresponding cisplatin-resistant cell lines (TE8R and TE10R) utilizing 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 performed 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 nM pre-miR miRNA precursor molecules of has-let-7c (#PM10436, Applied Biosystems) using SiPORT NeoFX (Ambion, Austin, TX) in six-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 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (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 μM). After incubation for 6 hours, the medium was changed into normal medium. 72 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-isopropanol. Absorbance was measured at 490 nm using a microplate reader (Bio-rad Laboratories, Tokyo).

Apoptosis Assay

Apoptosis was assessed by the flow cytometric detection of phosphatidyl serine externalization using Annexin V and propidium iodide staining (Bio Vision, CA, US). TE13 cells, after transfection with pre-let-7c and pre-miR negative controls, were treated with 40 μM 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 FACS Calibur flow cytometer (BD Bioscience, San Jose, CA).

ELISA assay
After 24-hours culture, the cells were exposed to 5 μM CDDP (mentioned above) or medium only. The supernatants were collected (24, 48, or 72 hr) and centrifuged. IL-6 protein level was measured using ELISA kits (#D6050, R&D Systems, Minneapolis, MN) 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 mM Tris, pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 1 mM phenylmethylsulphonyl flouride, 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 in this study: at 1:2000 for anti-human p-STAT3(Tyr705) antibody (#9145, Cell Signaling, Boston, MA), 1:2000 for anti-human STAT3 antibody (#9132, Cell Signaling), 1:2000 for anti-human p-Akt antibody (#9271, Cell Signaling), 1:2000 for anti-human Akt antibody (#4691, Cell Signaling), 1:2000 for anti-human Erk antibody (#4370, Cell Signaling), 1:2000 for anti-human Erk antibody (#4695, Cell Signaling), 1:10000 for anti-human β-actin (#A2066, Sigma-Aldrich), and 1:2000 for all secondary antibodies. Immune complexes were detected using the Detection Kit (GE HealthCare, Tokyo).

**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. Clinicopathological parameters were compared using the χ²-test.
and continuous variables were compared using Student’s $t$-test. Survival curves were computed using the Kaplan–Meier method, and differences between survival curves were compared using the log-rank test. A $p$ value <0.05 denoted the presence of a statistically significant difference. Statistical analysis was performed using the JMP Ver. 8.0 software.

**Results**

Altered expression of 15 miRNAs in cisplatin-resistant cells

PCR-based microarray analysis was performed to compare the expression of miRNAs in cisplatin-resistance cells and control cells using two pairs of cell lines; TE8/TE8R and TE10/TE10R. The miRNA microarray analysis in TE8/TE8R and TE10/TE10R 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 two cell lines. Among these 15 miRNAs, miR135a, miR-96, miR-141, miR-101, miR-146a, miR-489, miR-545 were up-regulated, while miR-99a, let-7b, miR-204, let-7c ,miR-202, miR-10a, miR-136 and miR-145 were down-regulated 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 performed quantitative RT-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, while SD and PD was observed in 35 and 6 patients, respectively. Thus, 33 (44.6%) patients were categorized as responder while the remaining 41 (55.4%) patients were categorized as non-responders. Expression of the 15 miRNAs was confirmed in the biopsy samples. We also divided the 74 patients of the training set into two groups based on 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 histopathological response.

With regard to the histopathological response in 74 patients of the training set, complete tumor regression (grade 3) and major tumor regression (grade 2) was observed in 3 and 9 patients, respectively, while minor tumor regression (grade 1) 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 histopathological response (Figure 1a,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 (Figure 1d). High expression of let-7b also tended to correlate with longer survival, but this tendency did not reach statistical significance (Figure 1c). We could not find significant relationship between let-7c expression and any clinicopathological 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 Figure 1a). To evaluate the biological 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. 1b). The MTT assay showed that let-7c-transfected cells were significantly more sensitive to cisplatin than
control cells. Furthermore, the IC50 of let-7c transfected cells was significantly smaller than that of the negative control (Figure 2a, 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 h, p=0.049, Figure 2c, 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 down-regulation of IL-6 mediated signaling pathway. This was based on Target scan and miRBase Targets database, which demonstrated 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 demonstrated that cisplatin activated IL-6 mRNA in esophageal cancer cells (Figure 3a). Next, we assayed IL-6 levels by ELISA. Cisplatin significantly increased the amount of IL-6 in the conditioned media (Figure 3b). Furthermore, phosphorylated-STAT3, which is downstream of IL-6, was induced by cisplatin in esophageal cancer cells (Figure 3c,3d).

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 (Figure 3e), and Annexin V assay showed that knockdown of IL-6 in esophageal cancer cells significantly increased the rate of apoptosis (Figure 3f, 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 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 (Figure 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 Erk, which are downstream of IL-6 (Figure 4b,4c). 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 (Figure 4d). In contrast, IL-6 expression was significantly higher in cancer tissue than in noncancerous tissue.
Moreover, IL-6 expression correlated inversely with let-7c expression in noncancerous tissue and esophageal cancer tissue (Figure 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 let-7c is associated with low chemosensitivity in patients with esophageal cancer. The 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 utility of miRNA expression for prediction of response to chemotherapy. Yang et al.\textsuperscript{(20)} performed 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 et al.\textsuperscript{(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 up-regulation 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 anti apoptotic member of the Bcl-2 family and known to induce apoptosis in cooperation with anticancer drugs that target Mcl-1, anti-apoptotic Bcl-2 protein (23). In oral cancer cells, let-7d negatively regulated EMT expression by targeting twist and snail and played an 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 et al (26) reported that autocrine production of IL-6 confers resistance to cisplatin and paclitaxel. Ilipoulos et al (18) reported that IL-6 plays a pivotal role in chemoresistance by inducing the conversion of nonstem cancer 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 et al. (27) showed that IL-6 secreted from endothelial cells after treatment with doxorubicine created chemo-resistant niche, and is involved in increased resistance to DNA damaging agents in paracrine manner. Indeed, we demonstrated 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 STAT-3 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 STAT-3, and to be involved in anti-apoptotic 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 phosphatidylinositol 3-kinase, mammalian target of rapamycin and phosphate and tensin homolog 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 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 et al.\textsuperscript{(18)} demonstrated 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 \textit{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 regimes that do not include cisplatin, because cisplatin–resistant cell line used in this study did not show resistance to 5-fluorouracil 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 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 demonstrated 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.
References


32. Plowman GD, Sudarsanam S, Bingham J, Whyte D, Hunter T. The protein


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

<table>
<thead>
<tr>
<th>microRNA</th>
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<th>TE10R/TE10 fold change</th>
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<td>miR-204</td>
<td>0.35</td>
<td>0.29</td>
</tr>
<tr>
<td>let-7c</td>
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<td>0.11</td>
</tr>
<tr>
<td>miR-202</td>
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<td>0.01</td>
</tr>
<tr>
<td>miR-10a</td>
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<td>0.06</td>
</tr>
<tr>
<td>miR-145</td>
<td>0.52</td>
<td>0.03</td>
</tr>
<tr>
<td>miR-136</td>
<td>0.54</td>
<td>0.002</td>
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</table>
Table 2. Relationship between the expression of 15 microRNAs and clinical response.

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Responders (n= 33)</th>
<th>Non-responders (n=41)</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
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<td>high/ low</td>
<td></td>
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<tr>
<td>miR-135a</td>
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<td>14 /27</td>
<td>0.640</td>
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<tr>
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<td>19/14</td>
<td>18/23</td>
<td>0.350</td>
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<tr>
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<td>19/14</td>
<td>18/23</td>
<td>0.350</td>
</tr>
<tr>
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<td>19/14</td>
<td>18/23</td>
<td>0.350</td>
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<td>20/13</td>
<td>17/24</td>
<td>0.160</td>
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<tr>
<td>miR-489</td>
<td>18/15</td>
<td>19/22</td>
<td>0.640</td>
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<td>18/23</td>
<td>0.350</td>
</tr>
<tr>
<td>miR-99a</td>
<td>15/18</td>
<td>22/19</td>
<td>0.640</td>
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<td>let-7b</td>
<td>22/11</td>
<td>15/26</td>
<td>0.019</td>
</tr>
<tr>
<td>miR-204</td>
<td>15/18</td>
<td>22/19</td>
<td>0.640</td>
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<td>14/27</td>
<td>0.005</td>
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<tr>
<td>miR-136</td>
<td>16/17</td>
<td>21/20</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Data are number of patients.
Legends for tables and figures

**Table 1**

Fold change in the expression of fifteen microRNAs in cisplatin-resistant cells compared with parental cells.

**Table 2**

Relationship between the expression of fifteen microRNAs and clinical response.

**Figure 1.**

Association of let-7b and let-7c expression with histological response and overall survival of patients treated with preoperative chemotherapy. (a-b) The expression of let-7b and let-7c was higher in patients with histological response of grade 2-3/grade 1 compared to those with grade 0 (let-7b: p=0.014/0.02; let-7c: p=0.032/0.025) (c-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).

**Figure 2.** Overexpression of let-7c increases chemosensitivity in esophageal cells. (a-b) The IC50 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 three experiments. *p<0.01.
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 hr exposure. (b) Cisplatin significantly increased the expression of IL-6 protein in supernatants of conditioned medium at 24, 48, and 72 hr exposure. (c) Western blots 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) Semiquantative analyses of expression of pSTAT3 and t-STAT3 in (c) by using densitometer. (e) The IC50 level of cisplatin in siIL-6 transfected cells is significantly lower than in negative control transfected cells. (f-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.

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 hrs. Data are mean±SD of three experiments. *p<0.01. (b) Western blots 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 three experiments with similar results. p-STAT3: phosphorylated STAT3, t-STAT3: total STAT3. p-Akt and p-Erk means phosphorylated Akt and Erk, respectively. (c) Semiquantative 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 chemoresitance. 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 pSTAT3, resulting in antiapoptosis and chemoresistance. Let-7 restores sensitivity to cisplatin through repressing IL-6/pSTAT prosurvival pathway by inhibiting directly IL-6 expression.
Fig. 1 Association of let-7b and let-7c expression with histological response (a,b) and overall survival of patients (c,d).
Fig. 2 Overexpression of let-7c increases chemosensitivity in esophageal cells.

(a) IC50 of CDDP (μM) for TE11 cell lines:
- Parent: 10.0 μM
- Mock: 7.5 μM
- Negative control: 8.0 μM
- let-7c transfected: 5.0 μM

(b) IC50 of CDDP (μM) for TE13 cell lines:
- Parent: 10.0 μM
- Mock: 7.5 μM
- Negative control: 8.0 μM
- let-7c transfected: 5.0 μM

(c) Annexin V analysis:
- let-7c transfected + CDDP: 0.69% live, 95.7% early apoptosis, 2.65% late apoptosis
- scramble + CDDP: 0.77% live, 96.2% early apoptosis, 1.45% late apoptosis
- let-7c transfected + CDDP: 0.60% live, 92.2% early apoptosis, 4.1% late apoptosis

(d) Graph showing increased chemosensitivity with let-7c transfected cells.
Fig. 3 Cisplatin activates prosurvival IL-6/STAT3 signaling pathway.
Fig. 4 Overexpression of let-7c represses IL-6/STAT3 prosurvival pathway after cisplatin exposure.
Clinical Cancer Research

Let-7 expression is a significant determinant of response to chemotherapy through the regulation of IL-6/STAT3 pathway in esophageal squamous cell carcinoma.

Keijiro Sugimura, Hiroshi Miyata, Koji Tanaka, et al.

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