

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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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 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 parent cell lines and examined the relationship between the miRNA expression and response to chemotherapy. *In vitro* assays were conducted 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 *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 interleukin (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. *Clin Cancer Res*; 1–10. ©2012 AACR.

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

nonresponders 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.

miRNAs are noncoding 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 upregulated, whereas miR-375, miR-27b, miR-203, miR-205, and let-7c are downregulated (11, 12). Recent studies also showed the involvement of several miRNAs in resistance to anticancer treatment including chemotherapy and radiotherapy. Giovannetti and colleagues (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 showed the involvement

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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/m², Adriamycin was administered at 35 mg/m² intravenously on day 1, and 5-FU was administered continuously from days 1 to 7 at 700 mg/m²/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% 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], as described previously (14). The cultured cells were exposed to 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 $12,000 \times g$ 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 $12,000 \times g$ for 10 minutes at 4°C , the supernatant was removed and washed with 1 mL of 75% ethanol. After centrifugation at $7,500 \times g$ 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'-CCTTCCAAAGATGGCT-GAAA-3', reverse primer, 5'-ATCTGAGGTGCCCATGCTAC-3'; GAPDH: forward primer, 5'-CAACTACATGGTTTACAT-GTTC-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 $\mu\text{mol/L}$). 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-isopropanol. 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 $\mu\text{mol/L}$ 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 $1 \times$ 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 $\mu\text{mol/L}$ 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

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 \pm 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.

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

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 |

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

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

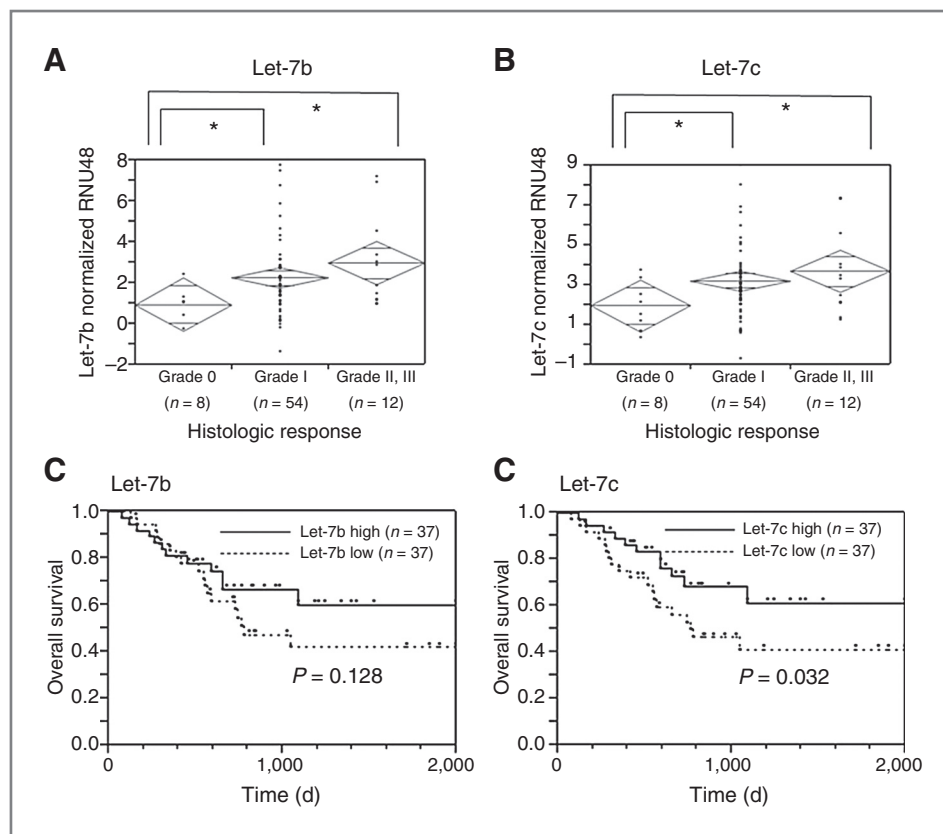
expression and any clinicopathologic parameter in patients who received preoperative chemotherapy followed by surgery.

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 IC₅₀ 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 ($P = 0.128$).



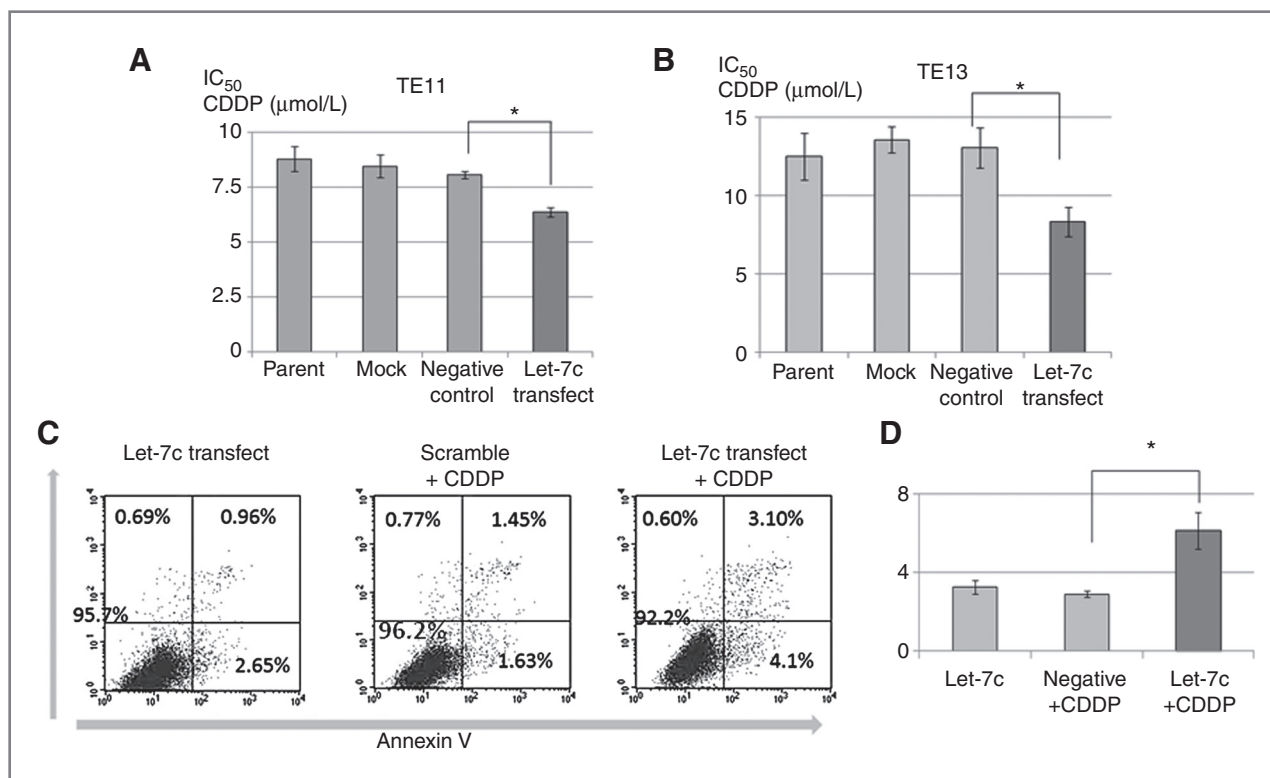


Figure 2. Overexpression of let-7c increases chemosensitivity in esophageal cells. A and B, the IC₅₀ levels of cisplatin in TE11 and TE13 esophageal cells were significantly lower in let-7c transfected cells than in negative control transfected cells. Data are mean \pm SD. *, $P < 0.01$. C, apoptotic 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 \pm 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

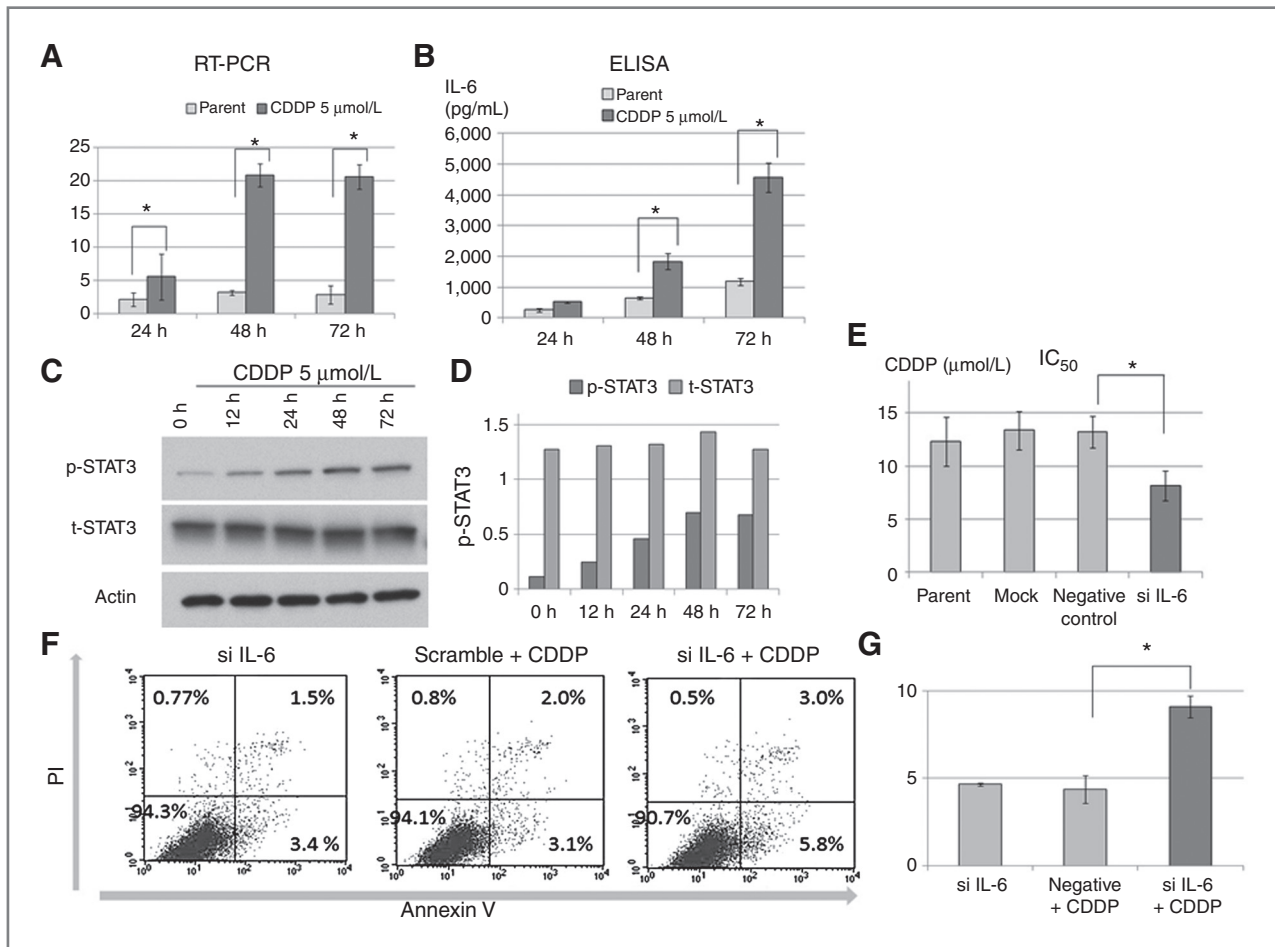


Figure 3. Cisplatin activates prosurvival IL-6/STAT3 signaling pathway. A, cisplatin significantly increased the expression of IL-6 mRNA in esophageal cancer cells at 24-, 48-, and 72-hour exposure. B, cisplatin significantly increased the expression of IL-6 protein in supernatants of conditioned medium 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 IC₅₀ 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 \pm 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

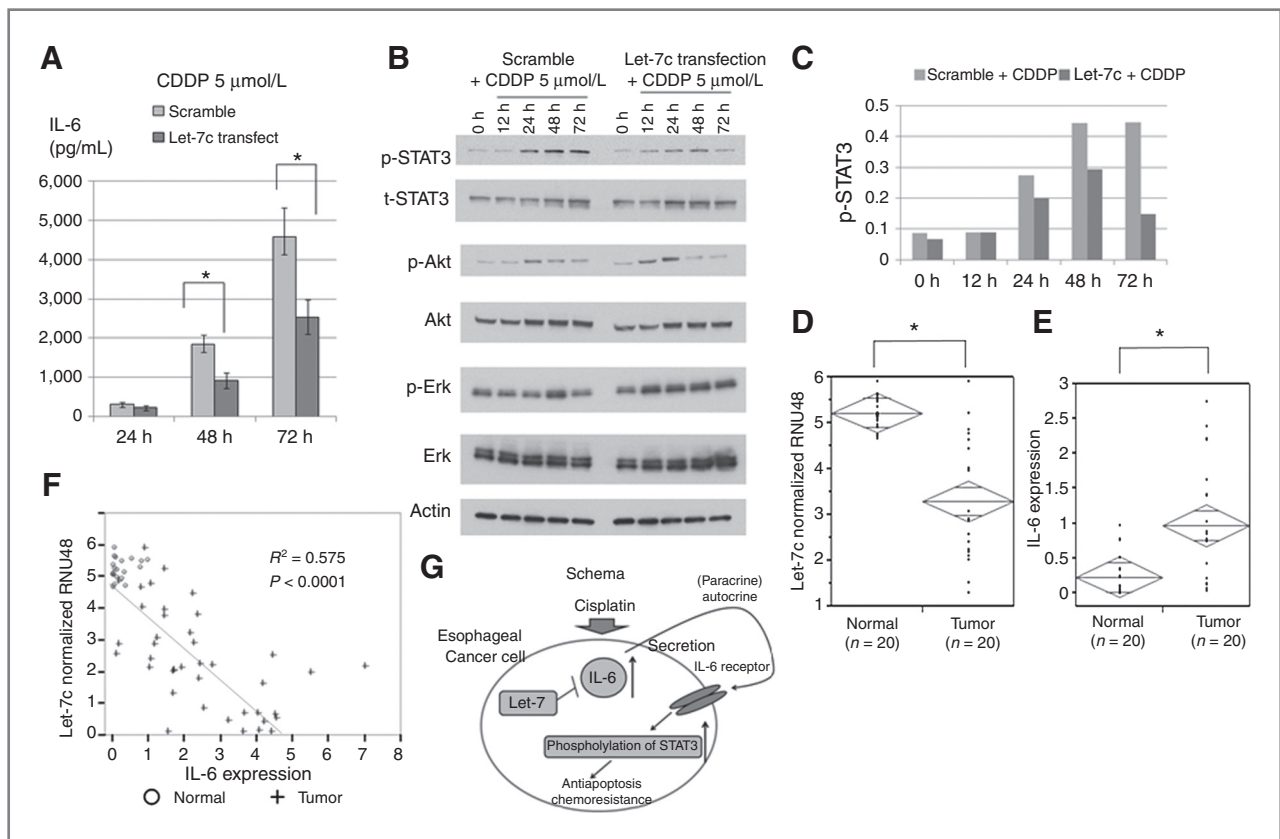


Figure 4. Overexpression of let-7c represses IL-6/STAT3 prosurvival pathway after cisplatin exposure. **A**, cisplatin significantly reduced IL-6 production in conditioned medium of let-7c-transfected cells compared with control cells at 48 and 72 hours. Data are mean \pm SD of 3 experiments. *, $P < 0.01$. **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

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- κ B, Lin28, let-7, and IL-6 form an inflammatory positive feedback loop. NF- κ B

induces Lin28 expression, leading to inhibition of let-7 and expression of the encoding IL-6. IL-6 can itself activate NF- κ B, 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 through 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.

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

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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

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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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